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JOURNAL OF SHELLFISH RESEARCH

VOLUME 23, NUMBER 1

APRIL 2004



The Journal of Shellfish Research
(formerly *Proceedings of the National Shellfisheries Association*)
is the official publication of the National Shellfisheries Association

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Journal of Shellfish Research

Volume 23, Number 1

ISSN: 0730-8000

April 2004

www.shellfish.org/pubs/jsr.htm



MAY 31 2004

**HONORED LIFE MEMBER
BRIAN L. BAYNE**

Take a passion for marine science, a piercing intellect, and a zest for life, and mix them all together under the heat of a Caribbean sun, and it's a heady brew! No question either about the talent for marine biology and molluscan physiology—any guy who kicks off his career with a single-author paper in *Nature* means business!

Brian, one of four brothers and a fourth-generation Trinidadian, was born in the West Indies in 1938. He grew up in and on the water, his favorite activity being scuba and spear fishing, in which his Dad was a pioneer. His love of the sea and interest in marine animals thus developed from an early age. He was sent to boarding school in Barbados at the tender age of 8. He matured rapidly when at 15 he persuaded his parents to send him to Ardingly College in the U.K. to complete his high school education. His boarding school years were successful academically, producing exam successes in chemistry, zoology, and botany, but less successful socially, producing a definite absence of the famed English reserve and stiff upper lip. Following a year in the "smoke" at Queen Mary College, University of London, he negotiated a transfer to University College North Wales, Bangor, to be closer to the sea. He graduated with a First in 1960 and promptly celebrated by marrying a softly spoken Welsh beauty, Marianne. He then took the long walk over the bridge from Bangor to Menai Bridge to obtain a Ph.D. in double quick time in 1963 under the benign aegis of Professor Dennis Crisp, working on the reproduction and larval ecology of mussel larvae.

Desirous of working with the best and expanding his scientific horizons, Brian and Marianne packed their bags and took off for the various corners of the world. Two years in Helsingør, Denmark, as a Churchill Foundation postdoctoral fellow working under Gunnar Thorson (University of Copenhagen) on the growth and metamorphosis of bivalve larvae, was followed by one year as a Visiting Research Fellow at the University of São Paulo, Brazil. Here Brian was introduced to respiratory physiology by Kjell Johansen (University of Washington, U.S.A.), so sparking off a life-long interest in the comparative physiology (functional biology) of bivalves.

Eventually, the lure of the tropics was overcome, presumably by the beer and "singing in the hills" of Wales, as Brian returned to spend two years as a Senior Research Fellow at the Shellfish Research Laboratory, Conway, working on the settlement and gregarious behavior of oyster larvae. This was followed by six years as a lecturer in the newly formed School of Biology at Leicester University, where residence in England's heartland was overcome by the construction of excellent recirculating seawater facilities and the choice of the hardy mussel *Mytilus edulis* as the experimental animal. During this period, a "tropical fix" was obtained via a Nuffield Research Fellowship, working six months at the University of Kuala Lumpur, Malaysia, and six months at Phuket Biologic Laboratory, Thailand, on the physiology of mangrove bivalves. In these early years, scientific productivity was matched by reproductive productivity, with the birth of daughters Julia in 1964 and Siân in 1968.

In 1973, Brian moved to Plymouth as a Principal Scientific Officer at the new Institute for Marine Environmental Research (IMER), working on the functional physiology and ecology of mussels. Stardom and relative fortune rapidly followed as Brian ascended the scientific and managerial ladder of fame. Promotion to Senior Principal Scientific Officer was followed by appointments to Director of IMER (1983), Director of the Plymouth Marine Laboratory (PML) (1988; formed from IMER and part of the Marine Biologic Association, U.K.), and finally in 1994, the dizzy heights of Director of the Center for Coastal and Marine Sciences (CCMS, comprising PML, Proudman Oceanographic Laboratory, and Scottish Association of Marine Sciences). Directorial qualities were tested to the full in orchestrating the delicate creation of PML and navigating the choppy waters of the genesis and functioning of CCMS. But enough was enough of managerial time consuming research time, and in 1997 Brian swapped the desk for the bench and became Research Professor and Deputy Director at the Center for Research on the Ecological Impacts of Coastal Cities, University of Sydney, Australia. He held this position until 2000, using oysters to extend work on the role of genetic variation on feeding and growth of bivalves, in the field of evolutionary physiology.

Science has been the major driving force in Brian's career, and his contribution to bivalve physiology, ecotoxicology, and adaptive biology is immense. His talents at the bench were manifest from the beginning in the rearing, handling, and experimentation of larval to adult animals. He sensed early on that bivalves were far more complex and interesting in their functional biology than was commonly appreciated, and that by applying relatively simple physiologic principles, it was possible to understand functional attributes. He was intrigued as to how such attributes could be used to analyze environmental quality, as well as understanding the adaptive features of the animals themselves. As different genetic stocks became available, so he researched the balance between environmental and genetic factors in determining phenotypic traits, leading to his current interest in the evolution of life histories.

He led substantial research groups from Leicester onwards, siring sixteen Ph.D. students, including a number of talented physiologists and the "odd" biochemist. Attracting a wide multidisciplinary team, the *scope for growth* and related approaches were developed, the legacy of which is that biologic effects measurements in mussels are today used worldwide in pollution monitoring. In later years, his bivalve research focused on the molecular bases underlying the physiologic advantages of genetic polymorphism, slower protein turnover being shown to explain faster growth. Studies on the interrelations between seston and bivalve feeding have helped define conditions for sustainable environmental shellfish aquaculture. Throughout his research career, Brian has benefited from and enjoyed collaborations with students, post-doctoral fellows, and fellow scientists, both at home and around the world. He has published some 180 primary papers, reviews and book chapters, and research funding has been obtained from many national and international grants.

Under Brian's directorship, IMER and PML continued to flourish into a major force in marine biology, attracting a host of talented staff and international visitors. In addition to the usual directorial qualities, this was achieved by Brian's own research pre-eminence and enthusiasm, plus a multidisciplinary breadth of knowledge that gave every scientist a fair shout and throw of the dice. Whatever the climate, fundamental studies were always encouraged. Inevitably, time for hands-on research was reduced, but ties were maintained by Visiting Research Fellowships to the State University of New York and Universities of Cape Town, South Carolina, and California at Davis. When schedules permitted, Brian would also be found back in the physiology laboratory, crouched over equipment, with concentrated disposition and faithful tea towel over the shoulder. And the first was always there! When a younger colleague quipped that it was good to see an old pro back at the bench, it was intimated where a scientific leg with scientific foot and boot attached might be heading if such flippancy persisted! And the same fire was taken to cricket matches with rival laboratories where no quarter was asked and none was given! In one infamous game, a foreign visitor, ignorant of the rules, was kept in conversation way outside his line of safety, while Brian snook up and dismissed him by whipping off his bails (for the non-cricketers amongst us, the aforementioned items refer to small horizontal wooden structures and not to a part of the man's anatomy)!

Such a distinguished career inevitably brings honors and accolades, and Brian has collected more gongs than you would find on a field marshal's chest. These include Honored Life Member Award of the National Shellfisheries Association, for which he is particularly pleased; Professorial Fellowships at the U.K. Universities of Plymouth, Sheffield, and Wales at Swansea; Honorary Fellowship at his *alma mater* University of Wales at Bangor; and Fellow of The Institute of Biology, the Zoological Society of London, The Royal Society of Arts, and The European Environmental Research Organization. Not surprisingly, word eventually reached the palace, and in 1998 he was awarded the Order of the British Empire for services to marine science. He has served on some thirty national and international committees, and seven international journals, including managing editor of the *Journal of Experimental Marine Biology and Ecology*.

Brian is an avid and accomplished single-handed sailor, and has spent much of his later years exploring the English Channel and further afield to Ireland, Scotland, and the Atlantic coasts of Spain. Avoiding the offshore passages, Marianne sensibly likes to join Brian once the boat has arrived at a pleasant cruising destination. I, along with other students, owe Brian much for being shown the correct way to do research, with thoroughness, no compromise, and integrity. I remember fondly the laugh to shake and warm your socks, the decisiveness (when it was time to go, it was time to go!), and the perceptive reasoning that cut straight to the heart of a debate amid the whirring of a cacophony of disparate arguments. Brian continues to work on the Australian study and to sail into various scientific and nautical ports. I can see him now, standing on the poop deck, gazing at the horizon, a broad smile alternating with a furrowed brow and intent peer that would send any perfect storm scuttlin' for cover! Sail on science boy, and congratulations on a career well done!

Dave R. Livingstone
Citadel Hill
Plymouth, U.K.



HONORED LIFE MEMBER JOHN HOOD RYTHER

John Hood Ryther was born in Newton, Massachusetts, in 1922. He attended Newton High School before joining the U.S. Army Air Force. He served from 1941 through 1945 and rose to the rank of Captain and Pilot. He subsequently attended Harvard University, receiving the degrees of A.B. (Cum Laude) in 1947, M.A. in 1950, and Ph.D. in 1951. John joined the staff of the Woods Hole Oceanographic Institution in 1951 as Research Associate. This was the beginning of a lifelong relationship during which he rose to the rank of Senior Scientist and served as Chair of the Biology Department and Director of the Coastal Research Center. John currently enjoys the status of Scientist Emeritus at Woods Hole. His prolific research career included many original and landmark contributions on primary productivity in the world's oceans, and in later years, numerous aspects of aquaculture. As his research in aquaculture grew John found an interested friend in the late Seward Johnson, and in 1983 John assumed the Director position of the Division of Applied Biology at the Harbor Branch Institution, Fort Pierce, Florida. He remained in that position until his retirement in 1987. John now resides with his wife Jean in North Falmouth, Massachusetts, where he enjoys his children, his growing grandchildren, and every opportunity to go fishing.

John's expertise and guidance were widely sought throughout his career. John served as a corporation member of both the Marine Biologic Laboratory at Woods Hole (1955–1981) and the Bermuda Biologic Station (1955–1962), including a period as President of the latter from 1961–1962. He served as a consultant to a wide variety of national bodies including the National Science Foundation, National Institutes of Health, Department of Interior, and Department of the Navy. He served as Director of the International Indian Ocean Expedition from 1963–1967, on the U.S. Marine Mammal Commission, the National Academy of Sciences, National Research Council, International Atlantic Salmon Foundation, International Council for the Exploration of the Sea, and on the editorial boards of numerous prestigious journals. Always in the forefront of international relations, John was a member of the U.S. Delegation of Oceanographers to the People's Republic of China in 1978 and the National Science Foundation Biologic Oceanographic Delegation to South Korea in 1979.

John's contributions to the world of aquaculture have been many, varying from macrophyte culture to waste-recycling aquaculture systems involving multispecies polyculture. Although this work was initiated over 25 years ago, variants on that same theme remain central to current efforts in locations around the world from the United States to Israel—a testament to the originality of his contributions. Of all his contributions, John is probably most familiar to National Shellfisheries Association members for two publications. The first contribution, published in 1962, is the development of f media in collaboration with fellow N.S.A. Honored Life Member Robert R. L. Guillard. Phytoplankton culture is an essential component of larval and juvenile culture as we currently know it, and the development of f media was a milestone in the march toward consistent production of food species for bivalve culture. The second is the major text on aquaculture co-authored with John E. Bardach and William O. McLarney. Published in 1972, this remains a valuable text even today.

I came to know John when serving as a postdoctoral fellow under his mentorship when I first arrived in the United States in late 1975. In addition to his brilliant intellect, John is a caring person with an infectious laugh and a love of life that he infuses in all who are exposed to him for any period. He retains an active interest in marine science and, as demonstrated to me on a recent visit to his Massachusetts home, still articulates challenging and innovative questions on a wide range of current research efforts from larval fish development on Georges Bank to macrophyte culture for industrial purposes. I consider myself very fortunate to have worked with him during my career.

Roger Mann
Virginia Institute of Marine Science
Gloucester Point, VA 23062

INFLUENCE OF FOOD SUPPLY ON POSTMETAMORPHIC GROWTH AND SURVIVAL OF HATCHERY-PRODUCED LION'S PAW SCALLOP, *NODIPECTEN NODOSUS* (LINNAEUS, 1758)

GUILHERME S. RUPP,¹* RAYMOND J. THOMPSON,¹ AND G. JAY PARSONS²†

¹Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, NL, Canada A1C 5S7;

²Fisheries and Marine Institute, Memorial University of Newfoundland, St. John's, NL, Canada A1C 5R3.

ABSTRACT Poor growth and low survival are often reported in hatchery-reared scallops early after settlement and metamorphosis. The effects of algal deprivation, substrate biofilm, and two algal cell concentrations (ca. 4.7×10^3 and 4×10^4 cells mL⁻¹) on growth and survival were investigated during 9 days after settlement in experiment 1, and the effects of the two algal concentrations were examined for 16 days post-set in experiment 2. In experiment 1, the presence of a biofilm on the collectors significantly enhanced postlarval settlement, but not growth. Five days after settlement, at a shell height of about 250 μ m, postlarvae fed a low algal concentration were significantly larger than postlarvae in the other treatments (unfed, biofilm-covered collectors and high algal concentration), which attained similar sizes. Nine days post-set, postlarvae fed at higher algal concentration were larger than postlarvae to which no suspended algae were supplied but were smaller than those cultured at low food concentration. Reserves accumulated during the larval stage were sufficient to sustain metamorphosis, limited shell growth, and high survival in the absence of exogenous food, giving no indications of food deprivation-induced mortality for at least 9 days post-set. In experiment 2, 10 days after settlement, postlarvae cultured at 4.7×10^3 cells mL⁻¹ were larger than postlarvae cultured at 4×10^4 cells mL⁻¹, but after 16 days post-set, postlarvae in both treatments attained similar shell height. Between 10 and 16 days after settlement (shell height ca. 300–400 μ m), feeding demand of postlarval *Nodipecten nodosus* significantly increased, as demonstrated by a sharp increase of gut pigment concentration, which was probably related to key events in the gill morphogenesis. Mortality, on the contrary, was higher at 4×10^4 cells mL⁻¹ than at 4.7×10^3 cells mL⁻¹. An alternative explanation to the high mortality of scallops often recorded early after metamorphosis in aquaculture settings, other than the depletion of endogenous reserves, is proposed. Food demand of postlarval *N. nodosus* is less than usually supplied in scallop hatcheries, and growth can be significantly increased early after settlement by adjusting the algal cell concentration.

KEY WORDS: biofilm, food, growth, hatchery, metamorphosis, *Nodipecten nodosus*, spat settlement

INTRODUCTION

Bivalve mollusks undergo important morphologic and physiologic changes upon completion of larval development, when the planktonic environment is abandoned and benthic life is assumed (Burke 1983). Metamorphosis is an energetically demanding process, and after the loss of the larval velum there is a cessation of filter-feeding capability until the postlarval ctenidia become functional (Bayne 1965, Hodgson & Burke 1988, Sastry 1965). During metamorphosis, the biochemical reserves used can be neutral lipids (Holland & Spencer 1973), proteins (Rodriguez et al. 1990), both proteins and lipids (Lu et al. 1999, Whyte et al. 1992), or proteins, lipids, and carbohydrates (Farias et al. 1998, Videla et al. 1998), although the energetic contribution of the carbohydrates is minor. Postmetamorphic shell growth of bivalves relies initially on endogenous reserves accumulated during larval life, and subsequent growth and survival are believed to depend on efficient acquisition of exogenous food before endogenous energy reserves are depleted (Whyte et al. 1992).

Though suspension feeding begins early in postmetamorphic oysters (Reid et al. 1992), which rapidly develop functional gills within a few hours after attachment (Baker & Mann 1994), in pectinids it is not clear when suspension feeding commences after metamorphosis. The anatomy of the gills of adult scallops differs

from that of other groups of bivalves due to the highly reduced latero-frontal cirri (Beninger 1991, Beninger & Le Pennec 1991), suggesting that scallops are not as efficient as other lamellibranchs in capturing small particles (<5–7 μ m) (Möhlenberg & Riisgard 1978, Palmer & Williams 1980). In addition, it is not clear if during postlarval stages, the efficiency in capturing small particles is also reduced. Gills of postlarval *Pecten maximus* at a shell height of 0.25–0.9 mm are believed to be partly functional (Beninger et al. 1994). The development of gills of postlarval *Placopecten magellanicus* is more protracted than in other bivalves, so that suspension feeding is probably inefficient in spat less than 1–2 mm in shell height (Veniot et al. 2003). According to Reid et al. (1992), effective suspension feeding in *Patinopecten yessoensis* starts one week after metamorphosis at a size of 400 μ m, when the supra- and infrabranchial chambers are formed. Some bivalves also use a transitional phase of pedal feeding, which allows detritus and benthic diatoms to be ingested, bridging the nutritional gap until suspension feeding becomes effective (Reid et al. 1992). Pedal feeding involves collection of epiflora by protrusion of the ciliated foot, which is then withdrawn into the mantle cavity, where benthic particles adhering to the cilia are transferred to the palps and then carried to the mouth (Reid et al. 1992). Pedal feeding is an important feeding mode for up to 12 wk after metamorphosis in the geoduck *Panope abrupta* (King 1986). It has also been documented in the scallop *Patinopecten yessoensis* at sizes <350 μ m, but in larger spat its nutritional contribution is not clear (O'Foighil et al. 1990, Reid et al. 1992). The uptake of dissolved organic matter (DOM) is also an alternative mode of nutrition, for bivalve larvae and spat, but its energetic contribution is considered low (Manahan 1983).

The period from metamorphosis to a size of 0.5 mm is critical

*Corresponding author. Current address: EPAGRI, Centro de Desenvolvimento em Aquicultura e Pesca, Rod. Admar Gonzaga 1188, Itacorubi, P.O. Box 502, Florianópolis, SC, 88034-901, Brazil. Tel.: +55 48 239 8040; Fax: +55 48 239 8028; E-mail: rupp@epagri.rcet-sc.br

†Current address: Fisheries and Oceans Canada, Aquaculture Science Branch, 200 Kent Street, Stn. 12W114, Ottawa, ON, Canada K1A 0E6.

for successful production of scallops in hatchery/nursery culture, where high mortalities have been recorded for the lion's paw scallop, *Nodipecten nodosus* (pers. obs.), as well as for other scallops (Abarca 2001, Bourne & Hodgson 1991, Bourne et al. 1989, Castagna & Duggan 1971, O'Foighil et al. 1990, Uriarte et al. 2001). Nevertheless, only few studies have focused on the influence of food on growth and survival of scallops within this size range (Nicolas & Robert 2001, O'Foighil et al. 1990). The causes of high mortality of postlarval scallops in hatcheries remain unclear. It has been suggested that depletion of endogenous reserves before exogenous food intake can meet the energetic demands of growth, and metabolism may explain these mortalities (O'Foighil et al. 1990, Whyte et al. 1992).

The body size at which suspension feeding becomes an important mechanism supporting shell growth during early postlarval life of *N. nodosus* is not known. Neither is it clear to what extent shell growth relies exclusively on endogenous food reserves, nor is it known whether pedal feeding is an important mechanism of food intake during early postlarval life.

The lion's paw scallop *N. nodosus* has great potential as an aquaculture species in Brazil, and due to insufficient wild spat collection (Rupp 1994), hatchery production of juveniles is the only source of spat for aquaculture development. Therefore, optimization of growth and survival during hatchery/nursery production will be essential for the establishment of scallop aquaculture. Considering the uncertainty about the feeding capabilities of early postlarval scallops, the objectives of the current study were to examine the influence of food availability on growth and survival of *N. nodosus* immediately after metamorphosis (0.2–0.4-mm shell height) and to determine the size at which availability of exogenous food begins to support growth and survival.

Postlarval growth and survival of *N. nodosus* were determined in two experiments when presence or absence of epiflora and suspended food at different concentrations were tested. It was hypothesized that if early postlarvae are not able to acquire food by suspension feeding due to delayed anatomic development of the gills, then growth would depend on endogenous food reserves or on food uptake by pedal feeding. If growth depended solely on endogenous reserves, then it would be identical in the presence or absence of exogenous food sources. Conversely, if postlarvae benefited nutritionally from bacteria and benthic microalgae by pedal feeding, then growth would be higher when they are on substrates covered by a biofilm, compared with unfed animals. Furthermore, if endogenous energy was depleted in scallops deprived of food, a significant mortality should occur. After the gills become functional, growth would be higher in postlarvae fed with algae. Additionally, postlarval growth, ingestion, and survival were determined at two algal concentrations.

MATERIALS AND METHODS

General Procedures

Larval Rearing

Larval rearing and postlarval culture experiments were undertaken at the Laboratory for the Culture of Marine Molluscs – Federal University of Santa Catarina (LCMM – UFSC), in Florianópolis, Santa Catarina State, Brazil. Broodstock (shell height, 11–12 cm) were collected in the wild by scuba diving and maintained in lantern nets off João da Cunha island (Porto Belo, SC) until transfer to the laboratory 14 days before the scallops were

induced to spawn. Scallops were maintained in tanks with flowing seawater at a temperature of 17–18°C and were fed a mixture of cultured microalgae (*Isochrysis* sp. (clone T-iso), *Chaetoceros calcitrans*, *C. muelleri*) at final concentrations of 4 to 6×10^4 cells mL^{-1} . Such conditions produce successful spawnings, resulting in large numbers of oocytes (Rupp et al. 1997). To induce spawning, scallops were first immersed for 1 h in UV-irradiated filtered seawater (UV-FSW) with a high concentration (ca. 5×10^5 cells mL^{-1}) of *Isochrysis* sp. (clone T-iso). They were then placed in flowing UV-FSW, and the water temperature was increased from 18 to 26°C over 3 h (Rupp 1994). As soon as gametes started to be released, individual scallops were separated in different containers. *N. nodosus* is a functional hermaphrodite, and the spawning scallops were frequently transferred to a new container to avoid mixing the different gametes, therefore minimizing self-fertilization. Fertilization was carried out using sperm and eggs released by different individuals (pooled from several individuals). Two batches of larvae were raised using scallop hatchery techniques (Rupp 1994, Uriarte et al. 2001) in 2000-L polyethylene tanks at densities starting at 10 larvae mL^{-1} and ending at 1–2 larvae mL^{-1} . Seawater was filtered (1 μm) and UV-irradiated, salinity was 33‰, and temperature was maintained at 23–24°C. Water was changed daily, and a mix (1:2:1) of *Isochrysis* sp. (clone T-iso), *C. calcitrans*, and *C. muelleri* (formerly *C. gracilis*, CHAGRA – Provasoli-Guillard CCMP Laboratory) in final concentrations of 1×10^4 to 3×10^4 cells mL^{-1} was supplied daily. Algae were cultured in 100-L plastic bags in Guillard "F/2"-modified media under fluorescent light. Bacterial proliferation was controlled by addition of chloramphenicol (1 mg L^{-1}) to the larval cultures.

Settlement

When larvae (pediveligers) displayed morphologic and behavioral characteristics indicating competence (conspicuous eyespot, well-developed foot, aggregation in clumps, forming mucous strands, and crawling behavior) and attained a mean shell length of ca. 200 μm (shell height, 185–190 μm), they were considered competent to undergo metamorphosis. Pediveligers were then retained on a 140- μm -mesh Nytex screen, subsampled, counted in a Sedgwick-Rafter chamber using a microscope, and then transferred to 100-L tanks (experimental treatments) at a stocking density of 1 larva mL^{-1} . Water was changed daily, and no antibiotic was used during the experiments. The collectors used for larval settlement were polyethylene screens (mesh size, 3 mm), which in previous experiments resulted in high settlement of spat and were convenient to manipulate for experimental purposes. The collectors were cut into rectangles (25 \times 50 cm), and the edges of the longer sides were tied together, so that they assumed a cylindrical shape. During water changes, postlarval scallops in the bottom of setting tanks were collected and observed under a dissecting microscope. When present, these assemblages were composed mainly of dead scallops and were removed. As well, any remaining unsettled larvae were removed from the experimental units 72 h from initiation of the experiment, so that variability in postlarval size was minimized. Scallops displaying conspicuous dissoconch shell were considered to have undergone metamorphosis and settlement.

Experiment 1

In experiment 1, four treatments were carried out in duplicate 100-L tanks. Treatments consisted of (a) no algae supplied, (b) no

algae supplied, but the collectors had previously been immersed in coarse-filtered seawater (sand filter ca. 100 μm) for 8 days to allow growth of an epiflora of bacteria and benthic microalgae, (c) a mixed algal diet consisting of *Isochrysis* sp. (clone T-iso) and *C. muelleri* (1:1) at a final concentration of 4×10^4 cells mL^{-1} , supplied daily after the water change, and (d) the same volume of algal culture supplied in treatment (c) but with a cell concentration reduced by 90% by gravity filtration using cellulose paper filter. This procedure resulted in a final algal concentration of 4 to 5×10^3 cells mL^{-1} (mean, 4.7×10^3 cells mL^{-1}) while keeping the volume of the culture and concentrations of dissolved nutrients the same as in treatment (c). Preliminary observations indicated that algal cells were intact after filtration. Algal cells were counted with a Coulter counter model Z1. Algal cultures in the exponential growth phase were supplied daily to treatments (c) and (d) at volumes of ca. 0.3–0.4 L of each species. Treatments (a), (b), (c), and (d) will hereafter be referred to as unfed (U), epiflora (E), high algal concentration (H), and low algal concentration (L), respectively. Swimming larvae were retained during water changes and returned to the experimental units until day 3 postsettlement, when they were removed, to reduce size variability of settled postlarvae. To determine shell height (distance from the umbo to the ventral margin of the shell), samples were taken of setting larvae and of spat at 3 days (1 collector/tank), 5 days (3 collectors/tank), and 9 days (3 collectors/tank) postsettlement, when postlarvae were detached from the collectors. Both sides of the collectors were then thoroughly brushed with repeated gentle movements inside plastic trays filled with filtered seawater. This procedure was repeated four times, until no more spat were retrieved from the collectors. Detached spat were then screened, separated from debris, and preserved in 4% buffered formaldehyde in filtered seawater for further counting and measurements.

Water temperature was maintained at 23°C ($\text{SD} \pm 0.5^\circ\text{C}$) and salinity at 33‰. Sampling to determine larval survival was carried out 5 and 9 days postsettlement (4 collectors/tank each day). Postlarval samples were preserved in 4% buffered formaldehyde in seawater. Shell heights were measured with an ocular micrometer using a binocular microscope.

Experiment 2

In experiment 2, treatments consisted of spat fed daily at concentrations equal to those in treatments (H) and (L), as previously described for experiment 1. Each treatment was carried out in duplicate 200-L tanks. General procedures were the same as for experiment 1, but extended until 16 days postsettlement. In this experiment, addition of algal diets started 3 days after settlement. Water temperature was maintained at 25°C ($\text{SD} \pm 0.5$) and salinity at 33‰. Postlarval shell height (2 collectors/tank each day) and survival (6 collectors/tank) were determined on days 3, 10, and 16 post-set. In addition, shell height (maximum dorso-ventral dimension perpendicular to the hinge) and shell length (maximum antero-posterior dimension, parallel to the hinge) were measured ($n = 120$) to determine the relationship between shell dimensions.

Gut Pigments

To determine ingestion of microalgae by the scallops, the pigment content in the guts of larvae and postlarvae was determined by the gut fluorescence technique (Yentsch & Menzel 1963), which is frequently used to determine pigment content in meso- and macrozooplankton ($>200 \mu\text{m}$) (Bamstedt et al. 2000). Larvae

($n = 100$ – 200) were sampled at settlement and at day 1 post-set. Postlarvae were sampled at 5, 10, and 16 days postsettlement, when they were detached from the collectors, counted, and thoroughly rinsed in FSW while retained in a 140- μm mesh screen (Nalgene filter). Pigments were extracted on 90% acetone at 4°C for 24 h. Fluorescence was determined with a Turner digital fluorometer (TD 10-AU), with readings taken before and after acidification with 10% HCl. The concentrations of chlorophyll *a* and phaeopigments (mainly phaeophytin and phaeophorbide) were calculated following Bamstedt et al. (2000). Because ingested chlorophyll *a* is rapidly degraded and converted into phaeopigments (Dagg & Wyman 1983), the total pigment content in the guts of postlarval scallops was calculated as the sum of chlorophyll *a* and phaeopigments.

Data Analyses

Data were analyzed with the SPSS statistical package (version 10). For experiment 1, nested ANOVAs ($\alpha = 0.05$) were first carried out to determine the significance of differences in postlarval shell height among collectors within a tank and among tanks within a treatment, which were both found to be not significant. One-way ANOVAs were then carried out after pooling the tank and collector data within a given treatment, with treatment as the main factor. Residuals were checked graphically for normality and homoscedasticity (Sokal & Rohlf 1995), and the assumptions for ANOVA were not violated. When significant differences were detected among means ($\alpha = 0.05$), a *post hoc* Tukey-B test was performed. In experiment 2, independent samples Student's *t* tests were carried out to determine differences between treatments. The number of postlarvae attached to the collectors was compared among treatments by one-way ANOVA and independent samples Student's *t* test for experiments 1 and 2, respectively. Linear regression was used to describe the relationship between postlarval shell height and length.

RESULTS

Experiment 1

Growth

The mean shell height of setting pediveligers was 187 μm ($\text{SE} = 0.8$) (shell length, 200.8 μm). Three days after settlement, mean shell height for all treatments ranged from 197.2 to 199.1 μm , and there were no significant differences among treatments (one-way ANOVA, $F = 0.038$; $\text{df} = 3,236$; $P = 0.99$) (Fig. 1). Five days after settlement, there were significant differences in post-larval shell height among treatments (one-way ANOVA, $F = 28.68$; $\text{df} = 3,716$; $P < 0.001$). Postlarvae fed at low algal concentration (L) attained a mean shell height of 247.2 μm ($\text{SE} = 2.2$), which was significantly greater than in the other treatments. Nine days after settlement, there were also significant differences in shell height among treatments (one-way ANOVA, $F = 155.3$; $\text{df} = 3,716$; $P < 0.001$). Postlarvae fed at low algal concentration (L) attained a mean shell height of 333.6 μm ($\text{SE} = 4.85$), which was again significantly greater than for postlarvae exposed to the other treatments (Tukey-B). Furthermore, postlarvae cultured at high ration (H) (mean shell height 284.5 μm , $\text{SE} = 3.35$) attained a significantly greater shell height than those in the treatments unfed (U) (mean shell height 246.1 μm , $\text{SE} = 2.61$) and epiflora (E) (mean shell height 241.8 μm , $\text{SE} = 2.27$) (Tukey-B). Postlarvae in the treatments (E) and (U) displayed similar shell heights (Tukey-B).

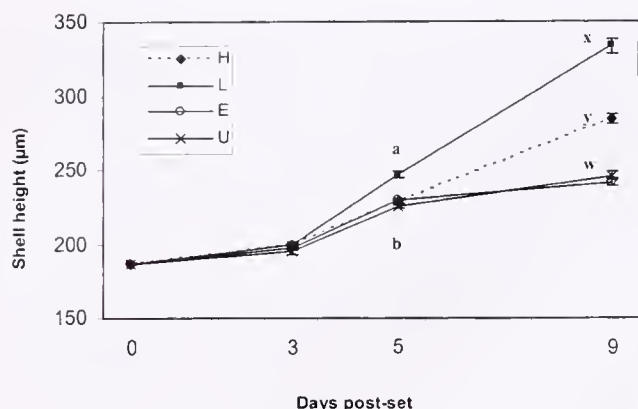


Figure 1. Shell heights (μm) of setting pediveligers (day = 0) and post-larvae 3, 5, and 9 days after settlement, cultured under four treatments (H = high algal concentration, L = low algal concentration, E = collectors covered by epiflora, U = no algae supplied) (mean \pm SE). (For each day, different letters denote significant differences.)

Daily growth rates from day 3 to day 9 were 22.61, 14.49, 7.43, and 8.15 $\mu\text{m day}^{-1}$ for the treatments L, H, E, and U, respectively.

Survival

The mean number of postlarvae attached to the collectors 5 days after settlement ranged from 166 (SE = 46) in the low food concentration (L) treatment to 369 (SE = 92) in the epiflora treatment (E) (Fig. 2). There were significant differences among treatments (one-way ANOVA, $F = 6.71$; $df = 3, 28$; $P = 0.001$). Collectors covered by an epiflora (E) had significantly more scallops than the other treatments, which were statistically similar to each other (Tukey-B). On average, 78 to 120% more postlarvae settled on the epiflora-covered collectors than on those in the other treatments. On day 9, there were also significantly more postlarvae on the collectors in the epiflora treatment. Overall, there was a decrease of about 26.8% in the number of spat attached to the collectors from day 5 to 9, and the percent loss of spat from the collectors did not differ among treatments (one-way ANOVA, $F = 0.57$; $df = 3, 7$; $P = 0.67$).

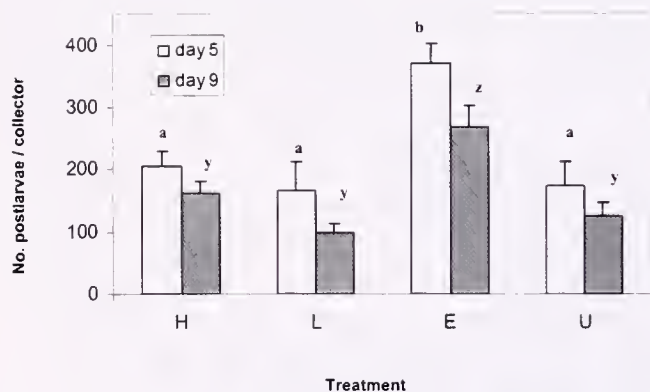


Figure 2. Number of postlarvae attached to collectors 5 and 9 days after settlement, cultured under four treatments (H = high algal concentration, L = low algal concentration, E = collectors covered by epiflora, U = no algae supplied) (mean \pm SE). (For each sampling date, different letters denote significant differences.)

Experiment 2

Growth

Setting larvae had a mean shell height of 190.7 μm (SE = 0.8) (shell length, 202.6 μm). Three days after settlement, mean post-larval shell height was 205.0 μm (SE = 1.67) and 208.1 μm (SE = 1.97) for the treatments of low algal concentration (L) and high algal concentration (H) respectively, which were statistically similar (t test, $t = -1.42$; $df = 238$; $P = 0.255$) (Fig. 3). On the other hand, 10 days after settlement, postlarvae from treatment (L) had attained a significantly greater shell height (mean = 320.2 μm , SE = 5.29) than those from treatment (H) (mean = 295.0 μm , SE = 4.07) (t test, $t = -3.76$; $df = 238$; $P < 0.001$). After 16 days, mean shell heights were 401.08 μm (SE = 7.24) and 403.17 μm (SE = 7.44) for treatments (L) and (H) respectively, which were statistically similar (t test, $t = 0.2$; $df = 238$; $P = 0.84$). Daily growth rates from days 3 to 10 were 16.02 and 12.85 $\mu\text{m days}^{-1}$, respectively, for treatments (L) and (H), and 13.48 and 18.03 $\mu\text{m days}^{-1}$ from days 10 to 16, respectively.

The relationship between shell height (SH) and length (SL) of postlarval *N. nodosus* had the following linear equation, $SH = 0.83 SL + 13.48$ ($n = 120$, $r^2 = 0.96$) (Fig. 4), which was highly significant (ANOVA, $F = 2488$; $df = 1, 119$; $P < 0.001$). This equation is provided as a useful means of comparison of shell dimension with other studies in which sizes are presented as shell length.

Survival

The mean numbers of postlarvae attached per collector in treatment H were 397.6, 237.5, and 176.3 for days 3, 10, and 16, respectively (Fig. 5). In treatment L, the mean numbers of post-larvae attached per collector were 482.5, 447.1, and 445.4 for days 3, 10, and 16, respectively. While there was no statistically significant difference in the number of spat/collector between treatments at day 3, in subsequent days the number of postlarvae in treatment L was significantly higher than in treatment H. In treatment L, loss of postlarvae from days 3 to 16 was 7% whereas in treatment H the loss was 55%.

Gut Pigments

Total pigments in the gut of larvae decreased from 108.7 ng ind^{-1} to 48 ng ind^{-1} after 24 h in the absence of suspended food.

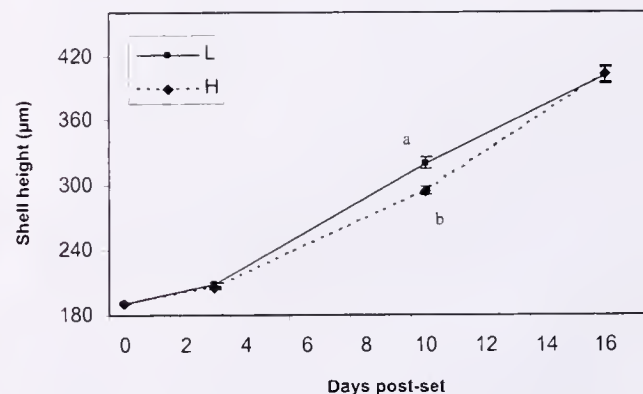


Figure 3. Shell heights (μm) of setting pediveligers (day = 0) and post-larvae 3, 10, and 16 days after settlement, cultured under two treatments (H = high algal concentration, L = low algal concentration) (mean \pm SE). (Different letters denote significant differences.)

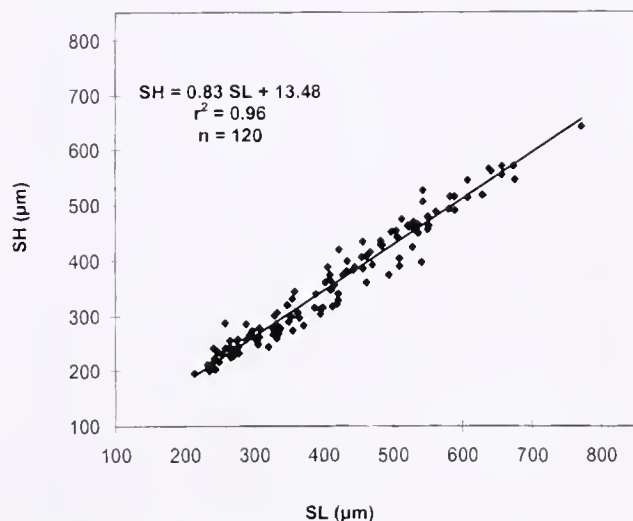


Figure 4. Relationship between shell height (SH) and shell length (SL) of postlarval *Nodipecten nodosus* ($P < 0.001$).

as algae were not supplied until 3 days postsettlement (Fig. 6). Five days after settlement, however, postlarvae had a higher pigment content in the gut than in the previous sample, both in treatment H (143.3 ng ind⁻¹) and in treatment L (170.48 ng ind⁻¹), which were similar (t test, $t = -1.39$; $df = 6$; $P = 0.1$). Ten days after settlement, postlarvae in treatment H displayed a slightly higher gut pigment content (316.11 ng ind⁻¹) than those in treatment L (212.42 ng ind⁻¹) (t test, $t = 1.99$; $df = 6$; $P = 0.048$). Subsequently, at 16 days post-set, the pigment content in postlarvae from treatment H (1307.6 ng ind⁻¹) was significantly higher in treatment L (462.5 ng ind⁻¹) (t test, $t = 4.54$; $df = 5$; $P = 0.003$).

DISCUSSION

This study focused on shell growth of *N. nodosus* shortly after settlement. Growth in juvenile bivalves occurs when energy acquisition exceeds metabolic demand to sustain basic physiologic processes, as there is no energy allocation to reproduction. In scallops, active suspension feeding on phytoplankton is the major form of food acquisition (Bricelj & Shumway 1991). During post-

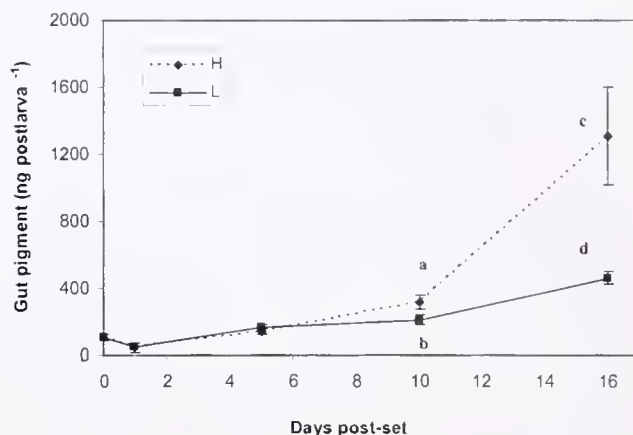


Figure 6. Pigment content (ng postlarva⁻¹) determined by gut fluorescence of postlarvae cultured under two treatments (H = high algal concentration, L = low algal concentration) after 1, 5, 10, and 16 days postsettlement (mean \pm SE). (Different letters denote significant differences.)

larval life, bivalves exhibit fast shell growth rates, which are not accompanied by a proportional increase in biomass. This growth pattern may be a protective adaptation of all bivalves, to reduce susceptibility to predation (Holland & Spencer 1973, Lu & Blake 1996, Whyte et al. 1992). In the current study, therefore, it is assumed that shell growth of postlarval scallops is maximized after the onset of acquisition and use of exogenous food.

There was a significant increase in postlarval shell height ($\Delta = 10$ to $13 \mu\text{m}$) from settlement to 3 days post-set in all treatments from experiment 1, regardless of the availability of exogenous food. The presence of a biofilm, suspended algae, or dissolved organic matter associated with the algal cultures did not result in faster shell growth than in the unfed control up to 3 days. These results demonstrate that shell growth of *N. nodosus* immediately after metamorphosis is not dependent on exogenous food, but fueled by endogenous reserves accumulated during larval life. This was also demonstrated in both treatments of experiment 2, when algae were not supplied for the first 72 h. Although the complete nutritional requirements of *N. nodosus* larvae remain unknown, the mixed diet consisting of the flagellate *Isochrysis* sp. (clone T-iso) and the diatoms *C. calcitrans* and *C. muelleri* (= *C. gracilis*) used during larval culture is widely used in scallop hatcheries, supporting significant growth and providing a balanced biochemical composition for larvae (Bourne et al. 1989, Farias 2001, Uriarte et al. 2001). In this study, *N. nodosus* pediveligers accumulated sufficient energy reserves to accomplish metamorphosis in the absence of exogenous food. In a study by Lu et al. (1999), *Argopecten irradians* was able to complete metamorphosis when deprived of food, and energy losses accounted for 57.9% of the total organic reserves. *Ostrea chilensis* pediveligers catabolized 65.5% of their energy reserves (Videla et al. 1998).

Five days after settlement, growth of postlarvae was significantly higher at low algal concentration (ca. 4.7×10^3 cells mL⁻¹) than in the other treatments. This indicates that postlarval *N. nodosus* at a shell height between 200 and 250 μm (days 3–5) can already capture and use phytoplankton as a food source; therefore, the gills are already capable of capturing particles. Furthermore, 9 days after settlement, postlarvae were larger when fed at low algal concentration than at high concentration (4×10^4 cells mL⁻¹). Algal cell concentrations fed to postlarval scallops in hatcheries

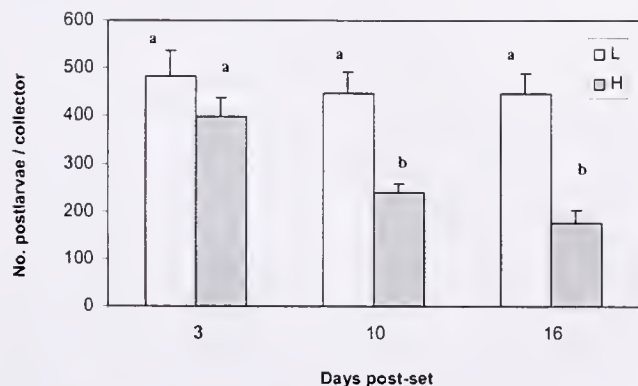


Figure 5. Number of postlarvae attached to the collectors 3, 10, and 16 days after settlement, cultured under two treatments (H = high algal concentration, L = low algal concentration) (mean \pm SE). (Different letters denote significant differences.)

usually range from 2.0 to 2.5×10^4 cells mL^{-1} (Bourne et al. 1989), 3.5×10^4 cells mL^{-1} (Dabinett et al. 1999), to 8.0 to 10×10^4 cells mL^{-1} (examples in Uriarte et al. 2001). The current results indicate that the feeding requirement of *N. nodosus* immediately after metamorphosis is very low, and that high algal concentrations hinder growth of postlarvae smaller than ca. $350 \mu\text{m}$. High algal concentration inhibits filtration in bivalve larvae (Gerdes 1983, Riisgard 1988, Sprung 1984). In juveniles and adults the gills are completely functional, and feeding activity can be controlled at high particle concentrations by discontinuous feeding behavior, reduction of clearance rate, or increasing pseudofaeces production (Bricelj & Shumway 1991). However, none of these mechanisms has been demonstrated in postlarval scallops smaller than 0.5 mm in shell height, in which the gills are not fully developed (Beninger et al. 1994, Veniot et al. 2003). The current study suggests that high algal concentrations may physiologically stress the scallops, preventing them from fully exploiting their growth potential. Riisgard (1991) postulated that low growth rates of bivalves recorded in laboratories may partly be due to unnaturally high algal concentrations, which lead to suboptimal conditions (valve closure, reduced metabolism, and reduced biosynthesis). Two other studies on postlarval scallops reported optimum thresholds for growth at low concentrations: 1.0 to 2.0×10^4 cells mL^{-1} for *Argopecten irradians* (Lu & Blake 1996) and 0.7 to 1.7×10^4 cells mL^{-1} for *Pecten maximus* larger than 0.5 mm (Nicolas & Robert 2001).

Nine days after settlement, postlarvae from the epiflora and unfed treatments had reached the same size and were significantly smaller than fed postlarvae. Surfaces immersed in seawater are rapidly colonized by bacteria and subsequently by diatoms and protozoans (Corpe 1970). Thus, the provision of biofilm-colonized substrates provided no nutritional benefit for *N. nodosus* for up to 9 days after settlement. These results suggest that pedal feeding, if indeed it does occur, makes a negligible contribution to growth of *N. nodosus*. It is also possible that the algae growing on the collectors were too large to be ingested by postlarval scallops or had no nutritional value. Microalgal assemblages on the collectors in this study were dominated by *Melosira* sp., a cosmopolitan non-planktonic chain-forming diatom (Hasle & Syvertsen 1997) that had individual cell size of ca. $25\text{--}30 \mu\text{m}$ but formed large chains ($>300 \mu\text{m}$). Although *Melosira* sp. is an abundant diatom in the gut contents of adult scallops (*Argopecten irradians*) (Davis & Marshall 1961) and *Placopecten magellanicus* (Shumway et al. 1987), it was probably not ingested by postlarval *N. nodosus* either due to the large size of the chains or absence of pedal feeding behavior. Within pectinids, pedal feeding has been reported only in *Patinopecten yessoensis* as a vestigial behavior, and its nutritional contribution is much less than suspended algae and limited to the first week after settlement (O'Foighil et al. 1990, Reid et al. 1992). If pedal feeding is a general mechanism in scallops, the suitability of different species of epibenthic algae that could be induced to grow on the collectors remains an interesting area for future investigations, which could contribute to optimization of postlarval growth.

Although the presence of epiflora on the collectors did not enhance growth of postlarval *N. nodosus*, it significantly increased settlement success, leading to 78–120% more postlarvae on the fouled collectors. Increased settlement induced by the presence of a biofilm has been demonstrated for some bivalves, including the scallops *Patinopecten yessoensis* (O'Foighil et al. 1990), *Chlamys hastata* (Hodgson & Bourne 1988), and *Placopecten magellanicus*

(Parsons et al. 1993), although in *Pecten maximus* a certain bacterial film did not influence settlement (Trittar et al. 1992). The effects of biofilms on settlement of *N. nodosus* have not previously been studied, and the current results have direct implications for the optimization of hatchery production. The immersion of the spat collectors in seawater to allow the formation of a biofilm is therefore a recommended hatchery practice.

There was an increment in shell height of $55\text{--}60 \mu\text{m}$ in the unfed and epiflora treatments, which was probably sustained by endogenous reserves, as the concentration of food particles in the water was negligible and tanks were covered to prevent entry of light, thus minimizing uncontrolled algal growth. Furthermore, mortality in these treatments did not differ from the fed group, suggesting no significant effect of food availability on survivorship during this period. Although survival beyond 9 days post-set was not followed, it is clear that the energy accumulated during the larval phase was sufficient to supply energy for maintenance for at least 9 days after settlement. Considering that acquisition and use of suspended food began 3 to 5 days after metamorphosis, the energy reserves accumulated during the larval period were sufficient to meet the demands of metabolism and growth of *N. nodosus* during the period in which there was no suspension feeding. In contrast to the rock scallop (*Crassodoma gigantea*), in which pediveligers had high mortality during 4 days of starvation (Whyte et al. 1992), *N. nodosus* not only survived, but also metamorphosed and displayed limited shell growth in the absence of algal food for at least 9 days post-settlement.

According to Whyte et al. (1992), the fatty acid profile of postlarval scallops *Crassodoma gigantea* indicate food assimilation at 25 days after metamorphosis, but their calculations suggest that before this, the acquisition of exogenous energy is not sufficient to meet the metabolic demand for growth, although earlier postlarvae were not analyzed. Postlarval mortality of *C. gigantea* was explained by insufficient acquisition of exogenous food soon after metamorphosis to meet the energetic demands of shell growth, which was postulated to be unimpeded by total energy depletion. The results of the current study, however, clearly indicate that shell growth of *N. nodosus* postlarvae is inhibited by the absence of suspended food compared with fed postlarvae, suggesting that energy acquisition from exogenous sources is a limiting factor for shell growth, and endogenous energy is not indiscriminately allocated to growth. Restrained growth in the absence of food may be a strategy to prolong survival under unfavorable conditions. If postlarval growth had been unimpeded until all endogenous energy was depleted, then mortality, not reduction in growth, would have been observed in the unfed treatment. The current results gave no indication of mortality induced by deprivation of suspended food for up to 9 days postsettlement, and food acquisition started well before endogenous energy was depleted. It is possible that the tropical scallop *N. nodosus* develops more rapidly than temperate species, such that the gills become functional earlier, being capable of capturing suspended particles at a smaller body size ($200\text{--}250 \mu\text{m}$ shell height) than *Patinopecten yessoensis*, in which effective suspension feeding begins at $400 \mu\text{m}$ (Reid et al. 1992), or than *Crassodoma gigantea*, two temperate water species. Indeed, Barré et al. (2002), comparing morphologic development of the cold-water *Placopecten magellanicus* and the warm-water *Argopecten irradians*, concluded that key events in gill development occurred at a smaller size in *A. irradians*, indicating inter-specific variability in gill ontogeny within pectinids.

Manahan (1983) demonstrated that filtrates from algal cultures (*Thalassiosira pseudonana*) contained large concentrations of dissolved free amino acids, which could be taken up by bivalves. In the current study, the same volume of algal culture media was added for both treatments of high and low food concentrations, but reducing the algal cell density by means of filtration in the latter treatment. This approach ensured that bacteria and concentrations of dissolved nutrients in the algal culture media, as well as exuded dissolved polymers and free amino-acids released by microalgae, which could have nutritional value for bivalves, were similar in both high and low food concentration treatments. Therefore, the only factor differing between treatments was the concentration of algal cells supplied, and any differential contribution of DOM among the treatments in which food was supplied was eliminated.

In experiment 2, after 10 days from settlement, postlarvae cultured at low algal concentration had shell heights significantly larger than those in the other treatments, consistent with experiment 1. However, 16 days after settlement, the shell heights of postlarval scallops were similar between treatments. The increase in the gut pigment content from settlement to day 5 post-set further confirms that at day 5, scallops were already able to ingest phytoplankton. Furthermore, at day 10, the pigment concentration was slightly higher in the scallops cultured at high algal concentration. As growth rates from days 3 to 10 were higher in the scallops cultured at low concentration, postlarvae used food more efficiently at low concentrations, as previously discussed. The current results suggest that there was an increase in food uptake around day 10 at ca. 300 μm shell height, but this increase in ingestion was not immediately reflected in growth, but in the subsequent period, when postlarvae cultured at high algal concentration displayed higher growth rates than those cultured at the low concentration. The sharp increase in the gut pigment of postlarvae from the high concentration treatment from days 10 to 16 suggests that at 300–400 μm shell height, there is a significant increase in feeding activity, which is probably associated with a critical stage in the gill development, as described for *Placopecten magellanicus* (Veniot et al. 2003) and *Pecten maximus* (Beninger et al. 1994). Postlarvae cultured at low algal concentration also displayed an increase in pigment content, but less than those at high cell concentration. At this point, the availability of food probably became limiting in the low food treatment, and ingestion of algae was therefore lower, but not resulting in immediate differences in shell height among treatments. Two nonexclusive hypotheses can be proposed to explain this result. First, because there is a lag between food acquisition, digestion, assimilation, and the shell growth response, differences in shell heights between treatments would only be apparent after day 16, and faster growth rates were indeed displayed by postlarvae at the high food treatment from days 10 to 16. Second, absorption efficiency tends to decrease at high food concentration, as demonstrated for other bivalves (Griffiths & King 1979, Navarro & Winter 1982, Thompson & Bayne 1974, Yukihiro et al. 1998). Thus, as the capacity of the digestive gland is exceeded, more organic material is rejected in feces. In this manner, even at lower food concentration, postlarval *N. nodosus* could maximize absorption of ingested food and display significant growth, despite lower algal ingestion. The current results suggest that food levels usually supplied to postlarval scallops in hatcheries are higher than the demand, and the high cell concentrations supplied may lead to reduced feeding rate and/or lower absorption efficiency, therefore resulting in a significant waste of cultured microalgae. The current study sets the stage for further

studies on the effect of cell concentration on the feeding physiology of scallop postlarvae, to maximize growth potential soon after settlement. Feeding early postlarvae at an optimum cell concentration of a suitable algal species could increase growth, significantly reduce the time to transfer to the sea-based nursery, and therefore reduce production costs.

Survivorship significantly differed between low and high food treatments in experiment 2, higher mortality being recorded at the higher food level. It is unlikely that the high cell concentration directly caused scallop mortality, but an excess of microalgae may have led to settlement of cells to the bottom of the tank, where they may have stimulated bacterial proliferation and reduced water quality. Nicolas & Robert (2001) also found the lowest mortality in postlarval *Pecten maximus* fed at the lowest food concentration. High bacterial concentration and accumulation of ectometabolites are detrimental to larval and postlarval bivalves (Elston & Leibovitz 1980, Loosanoff & Davis 1963, Prieur et al. 1990). Likewise, larval pectinids are highly susceptible to bacterial infections in hatcheries (Freites et al. 1993, Nicolas et al. 1996, Riquelme et al. 1995, Sainz et al. 1999), being more vulnerable to vibriosis than are larval oysters and penshells (Luna-Gonzales et al. 2002). The relatively immature immune system in larval pectinids compared with other bivalves may explain such differential susceptibility to pathogens (Luna-Gonzales et al. 2002). Although there is a lack of immunology studies of the early life stages of scallops, it is possible that early postmetamorphic scallops have a delayed development of the immune system compared with other bivalves and are therefore more susceptible to bacterial infection in hatcheries.

In conclusion, the current study demonstrated that the presence of epiflora on the collectors significantly increased settlement of the lion's paw scallop, but did not enhance postlarval growth. Furthermore, settlement, metamorphosis, and early postlarval growth were accomplished in the absence of suspended food, but 3–5 days (ca. 200–250 μm) after settlement, the availability of algae was an important factor determining growth of *N. nodosus*, and a low concentration (ca. 4.7×10^3 cells mL^{-1}) of *Isochrysis* sp. (clone T-iso) and *Chaetoceros muelleri* was more advantageous to growth and survival than 4×10^4 cells mL^{-1} . Between 10 and 16 days after settlement (ca. 300–400 μm), feeding demand of postlarval *N. nodosus* significantly increased, as demonstrated by increased gut pigment concentration, which was probably related to key events in the gill morphogenesis. The absence of exogenous food did not limit survival for at least 9 days after settlement, as ingestion of exogenous food started well before that. On the contrary, at high food concentration (4×10^4 cells mL^{-1}), mortality increased. Depletion of endogenous reserves before initiation of suspension feeding did not account for postlarval mortality. Alternatively, an immature immune system in early postlarval scallops, and therefore high susceptibility to disease, may explain the high postlarval mortality often reported in scallop hatcheries.

ACKNOWLEDGMENTS

This research was supported by a CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) Ph.D. scholarship to GSR, and the Brazilian Mariculture Linkage Program (BMLP), Canada. The Laboratory for the Culture of Marine Molluscs (LCMM), Federal University of Santa Catarina, is acknowl-

edged for providing experimental facilities and personnel assistance. We particularly thank Micheline M. de Bem for carrying out the larval cultures, Michella Fuck for helping out during the ex-

periments, and Drs. Pat Dabinett, Sandra E. Shumway, David Innes, and Don Deibel for valuable suggestions on an earlier version of the manuscript.

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BIOCHEMICAL COMPOSITION, REPRODUCTIVE ACTIVITY AND FOOD AVAILABILITY OF THE LION'S PAW SCALLOP *NODIPECTEN SUBNODOSUS* IN THE LAGUNA OJO DE LIEBRE, BAJA CALIFORNIA SUR, MEXICO

M. ARELLANO-MARTÍNEZ,^{1,*} I. S. RACOTTA,² B. P. CEBALLOS-VÁZQUEZ,¹ AND J. F. ELORDUY-GARAY¹

¹Centro Interdisciplinario de Ciencias Marinas, Instituto Politécnico Nacional, La Paz, Baja California Sur 23000, México; ²Centro de Investigaciones Biológicas del Noroeste, La Paz, Baja California Sur 23000, México

ABSTRACT: The relationships between the nutrient storage cycle, reproductive activity, and available food for the lion's paw scallop *Nodipecten subnodosus* were investigated during a one-year period in Laguna Ojo de Liebre, Baja California Sur, Mexico. Based on histologic data, this study showed that the reproductive cycle of *N. subnodosus* could be divided in four periods: (1) period of quiescence from December to March (with a small reproductive activity in February–March), (2) period of initial reproductive activity from April to May, (3) period of maximum ripeness from June to September, and (4) period of spawning from September to November. The seasonal changes in biochemical composition of somatic tissues of *N. subnodosus* at this locality indicated a clear nutrient storage and use cycle related to reproductive activity. However, the biochemical reserves of adductor muscle, digestive gland, and mantle were used differently over time. Proteins and acylglycerides could be transferred from the testis portion to the ovary portion during the ripening process. We showed that gametogenesis in *N. subnodosus* from Laguna Ojo de Liebre relied on stored reserves, as a consequence of the low food quantity (maximum 1.2 $\mu\text{g L}^{-1}$ chlorophyll *a*) and the low nutritional quality (maximum food index 17%) of the food available for suspension-feeders. The spawning of *N. subnodosus* in Laguna Ojo de Liebre coincided with the chlorophyll *a* maximum concentration, so it appeared to be triggered more by the phytoplankton abundance than by temperature.

KEY WORDS: food availability, *Nodipecten subnodosus*, nutrient transference, reproduction, scallop

INTRODUCTION

The reproductive cycle of marine bivalves is strongly related to nutrient storage and use cycles and environmental parameters such as water temperature and food availability (Giese 1969, Gabbott 1975, Bayne 1976). Gametogenesis is a process that requires energy, and the way to obtain energy differs among species (Park et al. 2001). Some species use the recently ingested energy from the seston (opportunistic species); others use the energy of substrates stored in various organs and tissues (digestive gland, adductor muscle, or mantle) through feeding prior to its gametogenesis (conservative species) (for reviews see Gabbott 1975, Bayne 1976, Barber & Blake 1991). In pectinids, the way in which the substrates are used, and the tissue or organ from which the associated energy is obtained, may vary among species (Ansell 1974, Comely 1974, Taylor & Venn 1979, Epp et al. 1988, Barber & Blake 1991, Martínez 1991, Mathieu & Lubet 1993).

Nodipecten subnodosus is a large pectinid species, reaching a maximum length of 218 mm (Félix-Pico et al. 1999). The lion's paw scallop supports a small commercial fishery only on its northern range of distribution because of its scarce presence in other areas of the Baja California peninsula. The lion's paw is an organism that reaches its commercial size in a relatively short time (Gutiérrez-Villaseñor & Chi-Barragan 1997). Its large adductor muscle is attractive for human consumption, a fact that has important implications for fisheries and aquaculture. However, only a few studies on growth (Carvajal-Rascón 1987, García-Domínguez et al. 1992, García-Pámanes et al. 1994, Barrios 1997, Ortiz-Cuel et al. 1997, Félix-Pico et al. 1999, Racotta et al. 2003) and reproduction (Reinecke-Reyes 1996, Gutiérrez-Villaseñor & Chi-Barragan 1997, Racotta et al. 2003) have been reported.

This study analyzes the seasonal variations in biochemical composition in a wild population of *N. subnodosus* and its relation to their reproductive activity and food availability.

MATERIALS AND METHODS

Animals

Samplings were done in the Laguna Ojo de Liebre (27°55'–27°35' LN and 114°20'–113°50' LW) located in the Mexican Reserve of the Biosphere "El Vizcaíno." From December 2000 to November 2001, 10 lion's paw scallops of 13–16 cm in shell height and 12–15 cm in shell length were collected monthly by divers at 7 m of depth. Scallops were transported alive in a cool and wet environment to lower their metabolism.

In the laboratory, the total and soft body weights and height (largest shell distance in an anterior to posterior orientation) of each scallop were registered to the nearest 0.1 mm using vernier calipers. Scallops were dissected to obtain gonads (without crystalline style and gut content), digestive glands, mantles, and adductor muscles, and their weights were registered and tissues were stored at –80°C for further biochemical analysis. Before freezing, a portion of gonad including both sexes was fixed in a formalin solution (10%) for further histologic analysis.

Indicators of Quantity and Quality of the Available Food

During the study period, seawater samples of the scallop-sampling area were collected using a Van Dorn bottle. The seawater samples were collected at 6.5 m of depth, close to the sandy bottom on which the scallops live. Water was screened through a 200- μm Nitex mesh to eliminate zooplankton and large particles before analysis (Park et al. 2001). For each analysis sample, in triplicate, a known volume of seawater was immediately filtered under gentle vacuum and in dark conditions through washed, precombusted, preweighed Whatman GF/F filters (47-mm diameter).

*Corresponding author. Fax: +55 (612) 1225344; E-mail: marellam@ipn.mx

The filters were washed with distilled water to remove residual salt.

Chlorophyll *a* was extracted on acetone, and its concentration was determined using the fluorometric method, as modified by Parsons et al. (1984).

The sum of carbohydrate, lipid, and protein concentration in the water, expressed as a percentage of the total seston, represents a useful index of the quality of the food available to suspension-feeders (Navarro & Thompson 1995). Food quality index (FI) was calculated according to Widdows et al. (1979), using the equation:

$$FI = \frac{FQ}{TPM} \times 100$$

where FQ is the food quantity ($\mu\text{g/L}$), and TPM is the total particulate matter or total seston ($\mu\text{g/L}$).

To calculate FQ, the major biochemical components of seston were analyzed: protein by the method of Lowry et al. (1951), carbohydrates by the phenol-sulfuric acid method (Dubois et al. 1956), and lipids by the sulfophosphovanillin method (Barnes & Blackstock 1973) using a commercial reactive and standard solutions (Merck, Darmstadt, Germany). FQ was then determined as the sum of the concentration of these components.

To calculate TPM, filters were dried at 80°C for 24 h and were reweighed after cooling in a desiccator. TPM was determined as the difference between the filter preweight (before filtering) and the dried filter weight.

Analysis of the Reproductive Activity

Transversal gonad sections including female and male portions were dehydrated in an ethanol series of progressive concentrations and embedded in paraplast X-tra. Sections (7 μm) were obtained and stained with hematoxylin-eosin (Humason 1979). Gametogenesis of *N. subnodosus* was categorized based on the qualitative characteristics of the gonad, separately for each sex, and by considering the five stages of gonadal maturation (undifferentiated, developing, ripe, partially spawned, and spent) proposed by Reinecke-Reyes (1996) for this species. The relative frequencies of the stages of gonadal development throughout the year were obtained with female and male stages combined, as there was not a difference in the temporality pattern between sexes.

To obtain quantitative values that represent the reproductive activity, the monthly gonadosomatic index (GSI) was calculated by dividing the gonad wet weight by the total soft body wet weight and by expressing the results as a percentage (Sastry 1970). Oocyte diameters were measured from digitized images of histologic sections using the SCAN PRO software (version 5.0, Systat Software, Inc., Richmond, CA). Oocytes sectioned through the nucleus were individually traced with the pointer, and the major and minor axis lengths were measured automatically by the software. Then, an average of both dimensions was calculated and considered as the estimated diameter, which was calculated monthly. At least 100 oocytes per scallop were measured. Individuals with few measurable oocytes and extensive phagocytosis ("spent" specimens) were not considered, using the criteria of Grant & Tyler (1983).

Additionally, indices of digestive gland (DGI), mantle (MI), and adductor muscle (AMI) were calculated as follows:

$$\text{Organ index} = \frac{\text{Organ weight}}{\text{Total soft body weight}} \times 100$$

Tissue Biochemical Analyses

To obtain a crude extract of digestive glands and gonads (female and male portions separately), 0.3 g of tissue was homogenized in 3 mL of cold saline solution (NaCl 35 ppt). This extract was used for all biochemical analyses. Muscle and mantle samples were processed in a different way when determinations were made (explained later for each case) because of their toughness.

Soluble protein determination was done by the Bradford (1976) technique, using commercial chromogen reagent (Sigma-Aldrich, St. Louis, MO) and bovine albumin serum (Sigma-Aldrich) as standard solution. Samples were first digested during 2 h with 0.1 N NaOH at a 1:10 dilution. For mantle and muscle, 0.02 g of tissue were directly digested in 2 mL of 0.5 N NaOH during 24 h. Then, 1 mL of Bradford reactive solution was added to 10 μL of digested sample, and absorbance was read at 595 nm.

Total carbohydrates were determined by the anthrone method (Van Anel 1965) after protein precipitation with trichloroacetic acid (TCA). For gonads and digestive gland, the crude homogenate was diluted 1:2 with 20% TCA, whereas samples of muscle and mantle (0.5 g muscle or 0.9 g mantle) were directly homogenized in 5 mL of 10% TCA. In both cases, samples were centrifuged at 4000 rpm at 5°C for 10 min. Then, 1 mL of anthrone solution (0.1% dissolved in 76% sulfuric acid) was added to 0.1 mL of supernatant and incubated at 86°C for 2 to 10 min, depending on the concentration of carbohydrates in each tissue and sampling period. The reaction was stopped by cooling at 4°C in an ice bath, and absorbance was read at 620 nm. Total carbohydrates were quantified as glucosyl units, using a calibration curve of glucose treated in the same way as particular samples, especially for the incubation times.

Total lipids were determined by the sulfophosphovanillin method (Barnes & Blackstock 1973) using a commercial reactive and standard solutions (Merck). A volume of 0.5 mL of concentrated sulfuric acid was added to 50 μL of crude homogenate of gonads and digestive glands, incubated at 90°C for 10 min and immediately cooled at 4°C in an ice bath. Then, 20 μL were mixed with 200 μL sulfophosphovanillin solution in a microplate, incubated for 40 min, and absorbance read at 540 nm with a microplate reader (Biorad 550, Tokyo, Japan).

Acylglycerides were determined by a colorimetric-enzymatic kit (GOP-PAP, Merck). The crude homogenate of gonads and digestive glands was centrifuged at 4000 rpm at 5°C for 10 min. Then, 20 μL were mixed with 200 μL commercial chromogen solution in a microplate, incubated for 20 min, and absorbance read at 490 nm with a microplate reader (Labsystems, Uniscan II). Lipids and acylglycerides were not analyzed in the muscle and mantle due to the lack of sensitivity of these methods on those particular tissues, which have a very low lipid content.

All the biochemical techniques were previously standardized for each tissue. The results of each biochemical constituent were expressed in mg g^{-1} of dry weight of each organ tissue.

Statistical Analyses

One-way ANOVA, followed by mean comparisons *post hoc* Tukey test were made to assess significant differences in biochemical composition and indices among months. Pearson product moment correlations were used between IGS and DGI, AMI, and MI, and the different biochemical variables.

Because GSI, DGI, AMI, and MI values are reported as per-

centages, they were arcsine transformed (Zar 1996) to reduce the dependence of the sample variance on the mean and to normalize the data distribution. Data are reported as means. STATISTICA for Windows (version 6.0) was used for analyses. The level of significance was pre-set at $P < 0.05$.

RESULTS

Gonad Maturation Process

Monthly qualitative assessment of the reproductive condition is illustrated in Figure 1. In December 2000 and from January to March 2001, most lion's paw scallops (between 75% and 100%) were inactive (undifferentiated and spent stages). The development stage was present in low percentage in January (12.5%) and with high incidence starting in April and increasing gradually to July (60–90%). Ripe scallops were found from June to November. The highest proportion of ripe scallops was observed in August (100%). Small proportions of ripe and partially spawned scallops were found in February and March. A massive spawning period was observed from September to November (25–75% of the population).

Oocyte Diameters

The mean oocyte diameter (Fig. 2) showed significant differences among months (one-way ANOVA, $P < 0.01$) with a clear seasonality (Tukey *post hoc* test). The minimum mean oocyte diameter (23.5 μm) was observed in January, and the maximum (51 μm) was observed in August. In December and March, the scallops were spent or undifferentiated, and thus oocyte diameter was not estimated.

Organ Indices

Significant differences (one-way ANOVA, $P < 0.01$) were observed for GSI throughout the year (Fig. 3). GSI values were low from December to March (5.5–7.4%); they began to increase gradually starting in April, reaching their maximum in August (26.6%), which was significantly different from other months. From September to November, the GSI values fell to levels similar to December 2000.

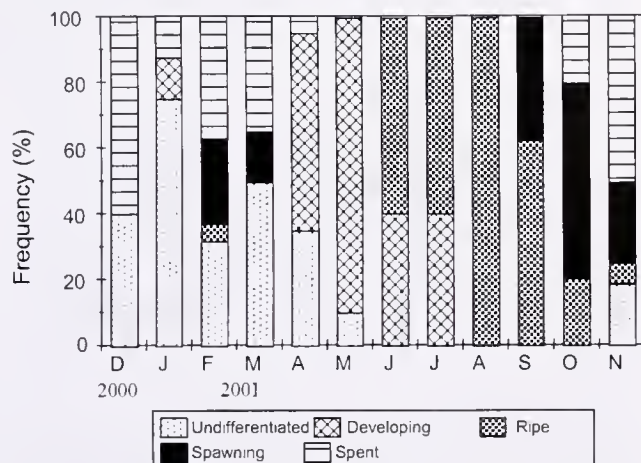


Figure 1. *Nodipecten subnodosus*. Monthly percent frequencies of the different gonad stages throughout the study period in Laguna Ojo de Liebre, B.C.S., Mexico. Male and female data were combined.

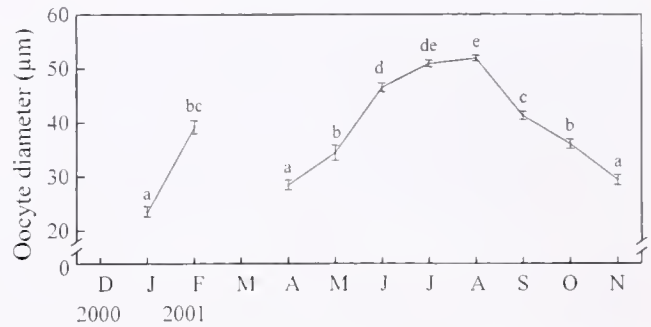


Figure 2. *Nodipecten subnodosus*. Temporal variation in oocyte mean diameter. Data was analyzed by one-way ANOVA, followed by Tukey test. Means not sharing the same superscript are significantly different. Bars correspond to standard errors.

DGI values changed significantly (one-way ANOVA, $P < 0.01$) throughout the year (Fig. 3). The highest value was observed in January (7.96%) and the lowest in October (5.79%) with intermediate values for the other months. DGI showed a significant negative correlation with GSI ($r^2 = -0.43$, $P < 0.001$).

Values of AMI were more than 32% of total biomass from

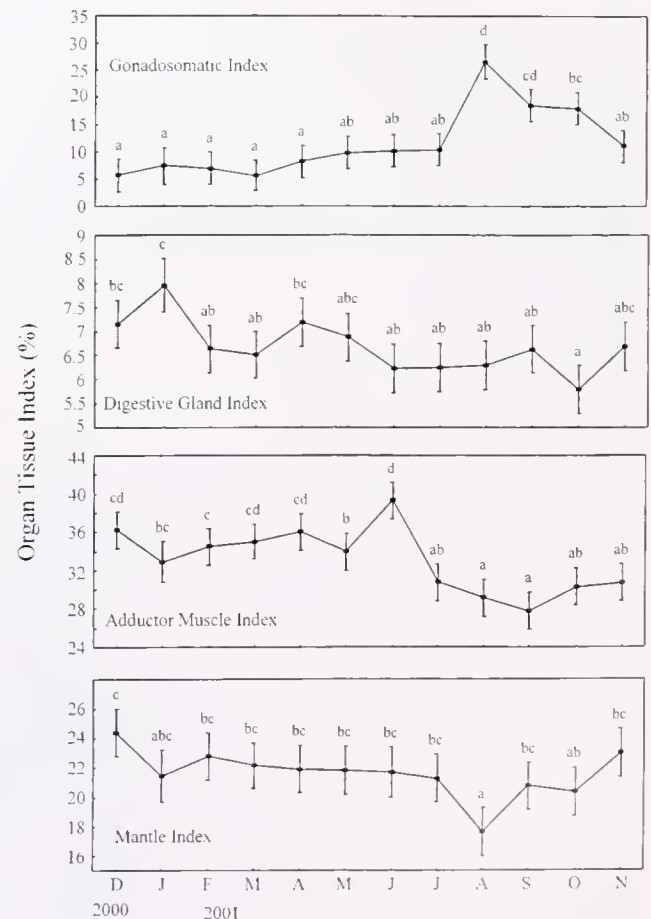


Figure 3. *Nodipecten subnodosus*. Temporal variation in the mean values of gonadosomatic index, digestive gland index, adductor muscle index, and mantle index. Data was analyzed by one-way ANOVA, followed by Tukey test. Means not sharing the same superscript are significantly different. Bars correspond to standard errors.

December 2000 to June 2001 and then significantly decreased (one-way ANOVA, $P < 0.01$) to values around 30% of biomass from July to November (Fig. 3). The AMI showed a significant negative correlation with GSI ($r^2 = -0.59$, $P < 0.001$).

Small, although significant (one-way ANOVA, $P < 0.01$) changes were observed for MI, which presented the highest value in December 2000 (24.2%) and the lowest in August 2001 (17.6%) (Fig. 3). The MI showed a significant negative correlation with GSI ($r^2 = -0.65$, $P < 0.001$).

Indicators of Quantity and Quality of the Food Available

Photosynthetic pigment concentration (chlorophyll *a* $\mu\text{g L}^{-1}$) and food index values in the Laguna Ojo de Liebre showed a similar variation throughout the year (Fig. 4). High chlorophyll *a* values (over $1 \mu\text{g L}^{-1}$) were observed in March and September–October in accordance with higher food index values (more than 11%). However, in September–October the higher values of chlorophyll *a* (1.19 and $1.2 \mu\text{g L}^{-1}$) were observed, but food index values were not the highest (under 12%).

Tissue Biochemical Composition

Protein

Protein was the major biochemical component of gonads. In the ovary portion, significantly lower values were observed from December to March. From April, the protein concentration increased gradually until significantly higher values were reached from June to October (Fig. 5). In the testis portion, a different pattern occurred (Fig. 5): protein increased gradually from January to April; then a decrease in protein concentration occurred from May to July, followed by a slight, though not significant, increase in August and September. Protein concentration in the ovary portion was significantly correlated to GSI ($r^2 = 0.55$, $P < 0.05$) but not in testis.

The digestive gland had a significantly lower concentration of proteins in April–May (Fig. 5). Higher values were observed in February and from June to August.

Protein levels in the adductor muscle were low from March to August (Fig. 5), whereas significantly higher values were observed from September to November.

In the mantle, the concentration of proteins decreased significantly from February to May (Fig. 5). Then, a significant increase was observed in July–August, followed by a significant decrease by September and then a further increase by November.

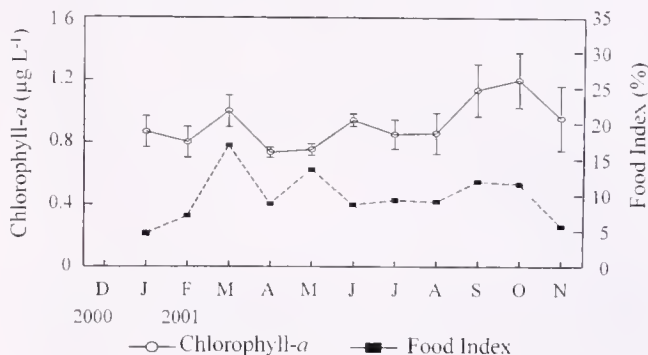


Figure 4. Temporal variation in chlorophyll *a* concentration and food index in the Laguna Ojo de Liebre, B.C.S., Mexico. Bars correspond to standard errors.

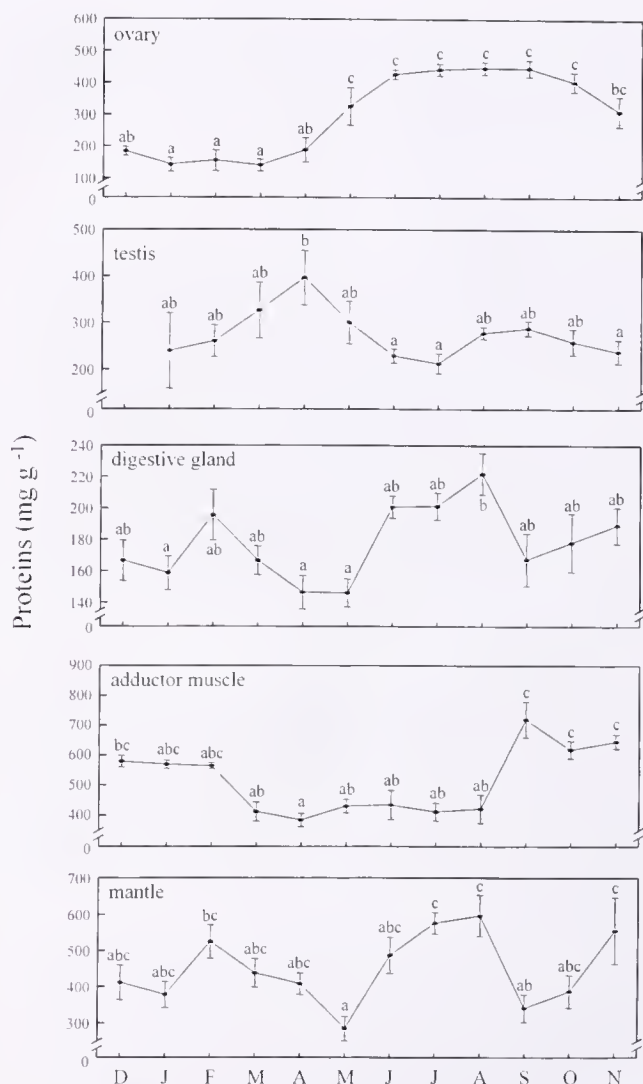


Figure 5. *Nodipecten subnodosus*. Temporal variation in the content of protein in ovary, testis, digestive gland, adductor muscle, and mantle. Data was analyzed by one-way ANOVA, followed by Tukey test. Means not sharing the same superscript are significantly different. Bars correspond to standard errors.

Carbohydrates

In the ovary portion, the significantly lowest values were present from December to April. By May, carbohydrates concentration increased gradually until September, when the significantly highest values were observed. Then, the concentration of carbohydrates declined in October–November (Fig. 6). In the testis portion, a similar pattern was observed, although the significantly highest concentration of carbohydrates was present in August, and the decline occurred in September (Fig. 6). The GSI showed a significant correlation with carbohydrates of ovary ($r^2 = 0.6$, $P < 0.05$) and testis ($r^2 = 0.48$, $P < 0.05$) portions.

The levels of carbohydrates in digestive glands showed a tendency to increase progressively throughout the year, with significantly higher values in August–September (Fig. 6). Carbohydrates in digestive glands showed a significant correlation to GSI ($r^2 = 0.41$, $P < 0.05$).

The concentration of carbohydrates in adductor muscle in-

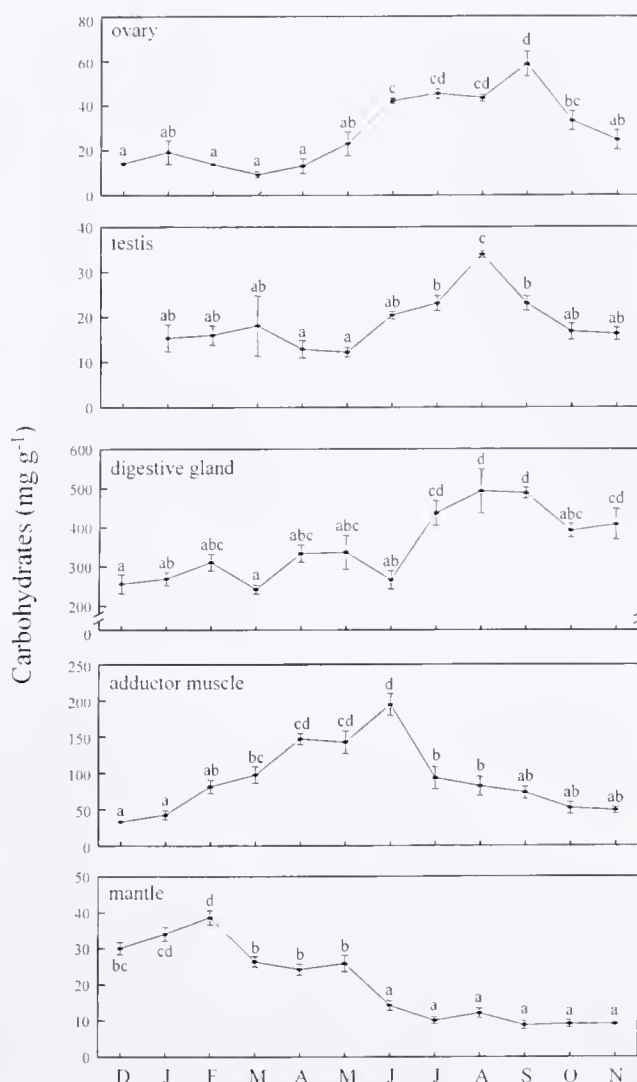


Figure 6. *Nodipecten subnodosus*. Temporal variation in the content of carbohydrates in ovary, testis, digestive gland, adductor muscle, and mantle. Data was analyzed by one-way ANOVA, followed by Tukey test. Means not sharing the same superscript are significantly different. Bars correspond to standard errors.

creased from December to June, when the significantly highest value was observed (Fig. 6). In July, a significant decrease was observed, and values continued to decrease until November.

The significantly highest concentrations of carbohydrates in mantle were observed from December to February (Fig. 6). Then, they decreased to intermediate levels from March to May and to the lowest values from June to November. Carbohydrates in mantle showed a significant negative correlation to GSI ($r^2 = -0.43$, $P < 0.05$).

Lipids

Lipids in the ovary portion showed a seasonal pattern. The lowest values were observed from December to April (Fig. 7). A significant increase was then observed from May, the highest values were attained from June to August, followed by a significant decrease from September to November. In the testis, lipids concentration did not change significantly throughout the year (Fig.

7). Concentration of lipids in the ovary portion showed a significant correlation to GSI ($r^2 = 0.47$, $P < 0.05$) but not in testis.

The concentration of lipids in digestive glands oscillated around high values from December to June with two peaks in February and May (Fig. 7). By July, lipids concentration fell significantly until November. Lipids showed a significant negative correlation to GSI ($r^2 = -0.38$, $P < 0.05$).

Acylglycerides

In the ovary portion, the lowest concentrations of acylglycerides were observed from December to March (Fig. 8). They increased from April, reaching significantly higher values from June to September, followed by a significant decrease in October and November. In the testis portion, acylglycerides levels showed a gradual but significant ($P < 0.01$) decrease from January to November (Fig. 8). Acylglycerides in ovaries were positively correlated ($r^2 = 0.51$, $P < 0.05$), whereas in testis they were negatively correlated ($r^2 = -0.33$, $P < 0.05$) to GSI.

The significantly highest concentrations of acylglycerides in digestive glands were observed from March to June (Fig. 8), whereas in the other months the values were significantly lower. Acylglycerides in digestive glands were negatively correlated to GSI ($r^2 = -0.41$, $P < 0.05$).

DISCUSSION

Gonad Maturation Process

Based on histologic data, the current study shows that the reproductive cycle of *Nodipecten subnodosus* may be divided into four periods: (1) period of quiescence from December to March (with a small reproductive activity in February–March), (2) period of initial reproductive activity from April to May, (3) period of

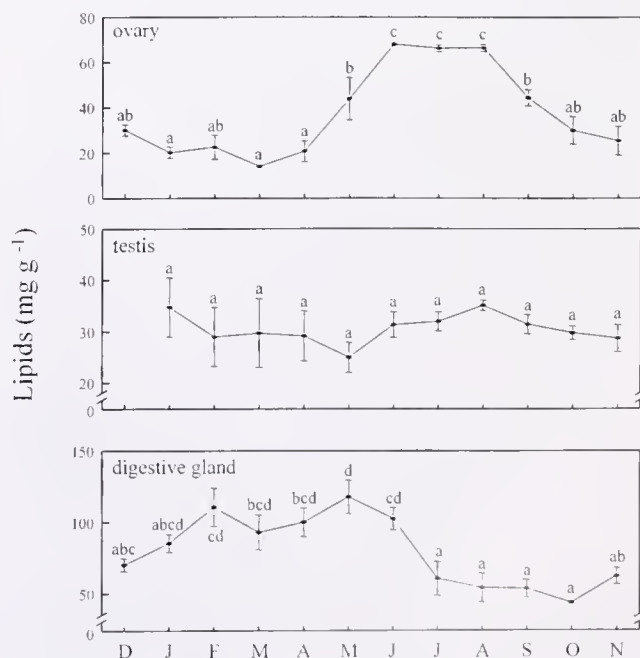


Figure 7. *Nodipecten subnodosus*. Temporal variation in the content of lipids in ovary, testis, and digestive gland. Data was analyzed by one-way ANOVA, followed by Tukey test. Means not sharing the same superscript are significantly different. Bars correspond to standard errors.

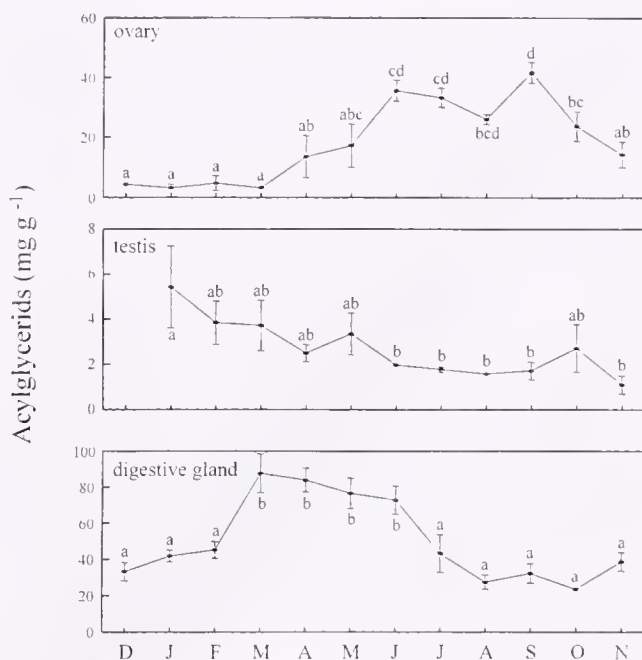


Figure 8. *Nodipecten subnodosus*. Temporal variation in the content of acylglycerids in ovary, testis, and digestive gland. Data was analyzed by one-way ANOVA, followed by Tukey test. Means not sharing the same superscript are significantly different. Bars correspond to standard errors.

maximum ripeness from June to September, and (4) period of spawning from September to November. This pattern is in accordance with previous studies of this species at the same locality (Reinecke-Reyes 1996) and at a southern locality (Racotta et al. 2003). However, in addition to the main spawning period, an earlier minor spawning is common in other species such as *Mya arenaria* (Brousseau 1987), *Pecten maximus*, *Pacopecten magellanicus*, and in the genus *Chlamys* (see Thompson 1977). Such a spawning was considered as facultative because it occurs only when environmental conditions are favorable (Brousseau 1987). In this sense, it is possible that *N. subnodosus* from Laguna Ojo de Liebre could begin a facultative spawning, as a slight reproductive activity was observed in February–March as indicated by the presence of some partially spawned scallops and by an increase in their oocyte diameter. However, in this period the IGS was low (<7%) as a consequence of the high proportion of spent (40%) and the presence of undifferentiated scallops (30%).

The positive correlations between all biochemical compounds analyzed in the ovary portion and the carbohydrates in the testis portion and GSI reflected their close relation to the reproductive activity. In relation to protein content in ovaries of pectinids, two patterns have been observed. Some studies showed an important accumulation of proteins in gonads during the ripening process (Barber & Blake 1981, Epp et al. 1988), whereas other studies reported that levels of protein in gonad tissue did not increase during gametogenesis (Thompson 1977, Couturier & Newkirk 1991, Pazos et al. 1996, Racotta et al. 1998, Ruiz-Verdugo et al. 2001, Racotta et al. 2003). However, these patterns probably are not a species-specific strategy, because *N. subnodosus* shows both patterns depending on the locality (Racotta et al. 2003 vs. current study). In the testis portion, a different variation in the protein content was found, as it decreased in accordance with the ripening

process in this study. In other functional hermaphrodite pectinids, contrasting results have been obtained. In *P. maximus*, the protein contents of the testis and ovary portions were nearly identical throughout the study (Lubet et al. 1987), whereas in *Argopecten purpuratus*, the protein level was higher in the female than in the male portion of the gonad in mature organisms (Martínez 1991). A possible explanation of our results is that proteins were transferred from the testis portion to the ovary portion during ripening and accumulated as yolk. Further work is necessary in functional hermaphroditic bivalves to test this hypothesis.

Despite that previous studies in pectinids have not reported an increase in the levels of carbohydrates in gonads associated with reproductive activity (Barber & Blake 1981, Couturier & Newkirk 1991, Martínez 1991, Pazos et al. 1996), our results indicated a significant increase during the entire maturation process and a decrease in the spawning period. It has been suggested that the accumulated carbohydrates in developing gonads can be used as an immediate available energy source for lipid synthesis or spawning (Racotta et al. 1998, Ruiz-Verdugo et al. 2001, Racotta et al. 2003) and also as precursors for lipid synthesis during the formation of gametes, which is known to occur in mollusks (Gabbott 1975, Lubet 1976, Barber & Blake 1985). Our results suggest that in *N. subnodosus*, the carbohydrates of testis could be used as an energy source for sperm motility.

The accumulation of total lipids and acylglycerides that we found in the ovary portion is an important and well documented process occurring during ripening in scallops (Barber & Blake 1991, Couturier & Newkirk 1991, Martínez 1991, Pazos et al. 1997, Racotta et al. 2003). These reserves are stored in ripening eggs and may subsequently be used by the larvae as an energy source during the first life stages of growth and metamorphosis (Holland 1978, Barber & Blake 1991, Couturier & Newkirk 1991). In contrast, in our results, acylglycerides levels in testis decreased significantly in parallel with the ripening process, and their concentration was negatively correlated to GSI. As for proteins, acylglycerides could be transferred from the testis portion to the ovary portion during the ripening process for the accumulation in gametes as yolk. In *P. magellanicus* (Thompson 1977, Robinson et al. 1981, Couturier & Newkirk 1991), *Chlamys septemradiata* (Ansell 1974), and *A. purpuratus* (Martínez 1991), the ovarian lipid content was higher than in the testis portion in ripe organisms.

Energy and Nutrient Supply for Gametogenesis

It is known that the mollusk digestive gland is responsible for nutrient storage and transfer of assimilated food to body tissues, and that the initiation of the oocyte growth phase is dependent on the accumulation and transfer of nutrient reserves from the digestive gland to the gonad (Sastri & Blake 1971, Gabbott & Bayne 1973, Vassallo 1973, Barber & Blake 1983, Pazos et al. 1997, Lodeiros et al. 2001). The variations of lipid, acylglyceride, and protein concentrations during gonad ripening observed in this study are in accordance with this transfer of energy and nutrients to the gonad for ripening in *N. subnodosus*. The rapid decrease in protein content between March and May (phase of early oocyte development) indicates that this substrate could be used for structural purposes during oocyte differentiation. On the other hand, the decline of lipids and acylglycerides between July and October indicated that they are used for yolk formation. Carbohydrates tend to increase slightly in the digestive gland, despite the development of gonads, thus suggesting that this organ did not transfer carbo-

hydrates to the gonad. In contrast to the apparent mobilization of proteins and lipids, Racotta et al. (2003) did not obtain any changes of these substrates in the digestive gland of *N. subnodosus* in Bahía Magdalena during gonad development.

The storage and mobilization of metabolic substrates from adductor muscle to the gonad during gametogenesis has been reported in bivalves, particularly for several pectinids such as *A. irradians* (Barber & Blake 1981, Epp et al. 1988), *Chlamys opercularis* (Taylor & Venn 1979), *P. maximus* (Comely 1974), *A. ventricosus* (Racotta et al. 1998), *A. purpuratus* (Martínez 1991), and *N. subnodosus* (Racotta et al. 2003). The depletion of proteins observed in adductor muscle from March to August suggests that lion's paw scallops use this reserve for supporting the whole process of gametogenesis. These results are in agreement with those reported for this (Racotta et al. 2003) and other pectinids, including *P. maximus* (Comely 1974), *C. opercularis* (Taylor & Venn 1979), *A. irradians* (Epp et al. 1988), *P. magellanicus* (Couturier & Newkirk 1991, Faveris & Lubet 1991), and *A. ventricosus* (Racotta et al. 1998). However, these results contrast with those of Barber and Blake (1981, 1985) and Brokordt et al. (2000), who suggest that protein reserves are used only to meet the cost of maintenance during final ripening and spawning.

In contrast, muscle carbohydrates increased from February to June, when oocytes started growing, but concentration decreased significantly during the final ripening phase (July to October) and during spawning (September to November). From the above, it is evident that *N. subnodosus* in Laguna Ojo de Liebre used carbohydrates stored in muscle to meet the final ripening and spawning needs.

The relative importance of the role of adductor muscle protein and carbohydrate reserves in supporting energy demands during reproduction in scallops is controversial. In some pectinids, the adductor muscle protein appears as the major energy reserve, which supplies the demand during gametogenesis (Barber & Blake 1985, Epp et al. 1988, Couturier & Newkirk 1991, Faveris & Lubet 1991, Pazos et al. 1997, Racotta et al. 1998, Racotta et al. 2003). In other species, the energy demands during gametogenesis are mainly supported by adductor muscle glycogen (Comely 1974, Taylor & Venn 1979, Barber & Blake 1981, Robinson et al. 1981).

The mantle tissue as a storage organ and its relation to gametogenesis has been observed in mussels (Gabbott 1975, Bayne et al. 1982, Mathieu & Lubet 1993) and scallops (Lodeiros et al. 2001). In the current study, a decrease in mantle proteins during early gonad development (March–May), as well as a further decrease in mantle carbohydrates during final ripening and spawning (June–November), may be considered as evidence that the lion's paw scallops store nutrients in the mantle to be transferred to the gonad during ripening.

The seasonal changes of biochemical composition in several somatic tissues of the lion's paw scallop *N. subnodosus* in this locality indicate a clear energy storage and use cycle strongly related to reproductive activity, as is common in other pectinids (Gabbott 1975, Barber & Blake 1991, Mathieu & Lubet 1993). This is further supported by significant negative correlations between several biochemical components in somatic tissues and GSI, as well as by the significant negative correlations of all somatic organ indices with GSI. The relation between organ indices and gonad development have been reported for scallops as an indicator of energy storage and use patterns (Barber & Blake 1981, Barber & Blake 1983, Villalejo-Fuerte & Ceballos-Vázquez 1996, Barrios 1997).

The current study shows that gametogenesis in *N. subnodosus*, at the locality of Laguna Ojo de Liebre, relies on previously stored reserves and thus behaves as a conservative species (Bayne 1976). In contrast, the results obtained by Racotta et al. (2003) for the same species, but at a southern locality (Bahía Magdalena), indicate that reproductive activity depends minimally on previously stored reserves, showing an opportunistic behavior (Bayne 1976). It is known that both strategies can be adopted by the same species to support gametogenesis, depending on food availability on a specific locality, which in turn depends on environmental variables that influence primary productivity (Barber & Blake 1991, Thompson & McDonald 1991, Luna-Gonzalez et al. 2000).

In this sense, Bahía Magdalena is considered a eutrophic lagoon with high primary productivity (Gómez-Gutiérrez et al. 2001), whereas our results show that Laguna Ojo de Liebre has a low food availability (maximum $1.2 \mu\text{g L}^{-1}$ chlorophyll *a*) year round, which agrees with previous works (Millan et al. 1987, Delgadillo-Hinojosa et al. 2002). Additionally, the food index (FI) values observed in our work (maximum 17%) can be considered as indicative of a low nutritional quality of the food available to suspension-feeders. Sites considered with a nutritionally dilute environment had FI values between 8% and 11% (Soniat et al. 1984, Luna-Gonzalez et al. 2000), whereas sites with a nutritionally rich environment had FI values as high as 55% (Navarro & Thompson 1995).

This could explain the contrasting results between our study and the previous work of Racotta et al. (2003). Then, the reproductive strategy (opportunistic vs. conservative) of *N. subnodosus* could be considered as an adaptation to food availability in a particular environment. A similar switch between both strategies was reported for *A. ventricosus* (Luna-Gonzalez et al. 2000) and *P. maximus* (Pazos et al. 1997), which use the available food in the environment, more than reserves, for gonadal ripening when the food is abundant, but they use the muscle reserves when the food abundance is poor.

Finally, changes in temperature, salinity, or photoperiod have been considered as physical environmental variables inducing spawning of bivalves (Mann 1979, Lubet et al. 1987). Park et al. (2001) mentioned that food availability also may have an effect on the initiation of spawning, and Starr et al. (1990) demonstrated that phytoplankton levels during blooms should be sufficient to induce spawning in *Strongylocentrotus droebachiensis* and *Mytilus edulis*. For other scallops, it has been suggested that spawning may be triggered by changes in temperature (Luna-Gonzalez et al. 2000) or phytoplankton blooms (Arsenault & Himmelman 1998). In *N. subnodosus* from Laguna Ojo de Liebre, the start of spawning coincides with the higher concentrations of chlorophyll *a*; then it is possible that the spawning of *N. subnodosus* was triggered by the higher concentrations of chlorophyll *a*, which, additionally, may ensure the food supply for larvae (Newell et al. 1982).

ACKNOWLEDGMENTS

We are grateful to Compañía. Exportadora de Sal, Sociedad Cooperativa Pescadores Unidos de Guerrero Negro, and Reserva de la Biosfera El Vizcaíno. This study was supported by the following projects: CGPI-200229, CGPI-2002072, PAC15, and SEMARNAT-CONACyT 2002-C01-0277. We are grateful to Instituto Politécnico Nacional for the grants (EDI and COFFA) to the authors. M. A. M. is a doctoral student-fellow of CONACyT. Our thanks also to Real English Agency for editing the manuscript.

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ENERGETIC REQUIREMENTS DURING GONAD MATURATION AND SPAWNING IN SCALLOPS: SEX DIFFERENCES IN *CHLAMYS ISLANDICA* (MÜLLER 1776)

KATHERINA B. BROKORDT^{1,2} AND HELGA E. GUDERLEY^{1,*}

¹Département de Biologie and Québec- Océan, Université Laval, Québec City, Québec G1K 7P4, Canada; ²CEAZA (Centro de Estudios Avanzados en Zonas Áridas), Casilla 599, La Serena, Chile, and Departamento de Ecología, Pontificia Universidad Católica de Chile, Alameda 340, Santiago, Chile

ABSTRACT Reproduction in scallops, as in many broadcast-spawning invertebrates, involves the production of a gonad that can become the largest tissue in the body. Gonad maturation leads to mobilization of macromolecular reserves from somatic tissues in many scallops. Because ovaries typically contain higher energetic contents than testes, we examined whether the energetic investment in gonad production and spawning and the impact on somatic tissues was greater in female than male Iceland scallops, *Chlamys islandica*. In males and females, maturation led to accumulation of carbohydrate and protein in the gonads, whereas lipids only accumulated in the ovary. In both sexes, the increase in gonad mass was accompanied by decrease of carbohydrate in muscle and the remaining tissues, but testes maturation was also paralleled by loss of carbohydrates and lipids from the digestive gland. Although spawning led to a greater energy loss in the female than in the male gonads (73 vs. 49 kJ in gonad energy content), less mobilization of somatic energy was observed during gametogenesis and spawning in females than males (14.5 kJ vs. 36.7). Most of the energy requirements for maturation and spawning in females must have been covered by feeding, whereas somatic reserve mobilization could have covered most of these costs in males. As in most scallop species, lipids account for a major part of the ovarian energy content. Direct deposition of dietary lipids in the eggs could minimize the impact on somatic tissues of female scallops. The costs of protein synthesis during testes maturation could decrease the aerobic scope available for feeding by males. In light of our previous demonstration of the impact of gonad maturation in *C. islandica* on recuperation from exhaustive exercise, our results suggest that the survival of males may be decreased more by their reproductive investment than that of females. The bioenergetic strategies of female scallops seem to favor survival and hence future reproduction more than those of male scallops.

KEY WORDS: biochemical reserves, *Chlamys islandica*, reproduction, reproductive cost, scallops, sex differences

INTRODUCTION

Intraspecific variation in how the reproductive cycle of scallops affects the mass and biochemical composition of body components has been attributed to the timing of gonad proliferation (Shafee 1981), to the habitat of the populations under study (eg. depth, protected or exposed) (MacDonald & Thompson 1986, Bricelj et al. 1987, and Epp et al. 1988), to the size or age group examined (Epp et al. 1988 and Martínez 1991), to the abundance and quality of the food supply, and to temperature (Shafee 1981, MacDonald & Thompson 1986, and Martínez et al. 2000). However, little is known about differences in reproductive investment between male and female scallops. In hermaphroditic scallops, the question of the relative investment in the male and female portion of the gonad is intriguing (Faveris & Lubet 1991), but marked qualitative and quantitative differences in reproductive investment of males and females are likely in species in which sexes are separate.

The cost of reproduction is typically believed to be higher in females than in males, given the large size of the egg relative to the sperm. Nuptial gifts, territorial defense, and paternal care of offspring can make the cost of reproduction higher for males than females. However, in broadcast spawning scallops, such behavioral considerations are unlikely to modify the cost of reproduction. The fact that the gonad can be the largest organ in scallops clearly indicates high investment in reproduction (Barber & Blake 1991), but whether the impact of this investment varies between males and females is an open question. The little that is published about sex differences in reproductive investment indicates that male and female scallops can differ in the size of the gonad (Couturier &

Newkirk 1991) and although the mature ovary and testis have similar protein contents, the ovary is richer in lipid (Couturier & Newkirk 1991 and Brokordt et al. 2000a). Various studies have examined reproductive investment in scallops, but most present pooled data for males and females. If investment in a given reproductive cycle decreases individual survival, it would reduce future reproductive success and thereby decrease fitness. Thus, reproductive investment represents a compromise between current and future reproduction. These compromises will differ between males and females if their equations linking reproductive investment with reproductive success and individual survival differ.

Mobilization of somatic reserves in support of reproduction should vary as a function of reproductive investment and of food availability. Mobilization of glycogen from the adductor muscle in support of gametogenesis and spawning occurs even when food availability is high (see Barber & Blake 1991 for review). In the Iceland scallop, *C. islandica*, and in the tropical scallop, *Envolva ziczac*, glycogen mobilization during gonad growth is accompanied by decreases in muscle activities of enzymes in energy metabolism and in the capacity to recover from exhaustive exercise (Brokordt et al. 2000a and Brokordt et al. 2000b). In *Argopecten purpuratus*, the decrease of muscle glycogen during gonad maturation also coincided with a marked decrease in escape capacities (clapping and recovery, K. Brokordt, unpubl. data). This suggests that mobilization of somatic reserves in scallops may decrease individual survival during attack by predators. In the black scallop, *Chlamys varia*, gametogenesis and spawning occur during periods of high and low food availability (spring and autumn, respectively), and only the autumn reproduction leads to extensive mobilization of protein, lipid, and carbohydrate from somatic tissues (Shafee 1981). In a comparison of two successive reproductive cycles in *E. ziczac*, recuperation from exhaustion was more im-

*Corresponding author. E-mail address: Helga.Guderley@bio.ulaval.ca.

paired in the first cycle in which reproductive investment was high while food availability was low. Muscle protein levels only declined during the first cycle, whereas muscle glycogen and enzyme levels declined during both cycles (Brokordt et al. 2000b). In these scallop species, gametogenesis and spawning consistently lead to depletion of muscle glycogen, but their impact on other somatic reserves and recuperation from exhaustion (in the case of *E. ziczac*) varies according to food availability and the extent of reproductive investment.

In this study we examined whether male and female Iceland scallops, *C. islandica*, differ in their reproductive investment and somatic reserve mobilization during a reproductive cycle. The greater lipid levels in mature ovaries (Brokordt et al. 2000a) suggest that females invest more energy in reproduction than males. However, as the synthesis of lipids may be less costly than that of proteins, the cost of spermatogenesis may exceed that of oogenesis. In the northern Gulf of St. Lawrence, a natural population of *C. islandica* shows marked and rapid gonad maturation during the spring increase in phytoplankton followed by a precipitous and synchronous spawning (Arsenault & Himmelman 1998). We sampled sexually mature males and females in a narrow size range (shell height = 79–81 mm) while their gonads were immature (May 30), mature (July 20) and after spawning (August 14). The mass and biochemical composition of the body components were measured and converted to energetic units using calorific coefficients to assess whether males and females differed in their reproductive investment and in its impact on somatic reserves. We measured carbohydrate, protein and lipid levels in the gonads, digestive gland, remaining tissues, (mantle, gill, foot, etc) and protein and glycogen levels in the adductor muscle. Because lipid levels in the adductor muscle are below 5% of dry mass (Couturier & Newkirk 1991) and typically change little throughout the reproductive cycle, they were not measured.

MATERIALS AND METHODS

Sampling, Animals, and Tissue Analyses

We studied the population of *Chlamys islandica* in the Mingan Islands (50°14'N, 63°36'W) in the northern Gulf of St. Lawrence, eastern Canada. Adult scallops (height 79–81 mm), 15 females and 15 males, were collected by SCUBA diving at 37 m in depth on May 30, July 20, and August 14, 2000. For both females and males, these sampling dates corresponded to when the gonads were immature, completely mature, and spawned. At each sampling period, we determined the shell height and wet and dry masses (oven-dried for 48 h at 80°C) of four body components: gonad, muscle, digestive gland, and the remaining tissues (mantle tissue, gills, excretory organ, foot, etc., henceforth referred to as remaining tissues). Tissues were frozen at -20°C, transported frozen to Université Laval, and then stored at -70°C until biochemical analysis.

Biochemical and Energetic Analyses

We determined the carbohydrate content in the female and male gonads, digestive gland, and remaining tissues using the phenol-sulfuric acid method of Dubois et al. (1956), as modified by Martínez (1991), with mussel glycogen as the standard. For the adductor muscle we determined the glycogen content by enzymatic hydrolysis with amyloglucosidase as described by Keppler and Decker (1974). The protein content in gonads, muscle, digestive

gland, and remaining tissues was determined using the bicinchoninic acid method of Smith et al. (1985), with bovine serum albumin as the standard. We determined the lipid content of all tissues (except for muscle) by extraction with chloroform-methanol according to Folch et al. (1957) and colorimetric determination with phosphovanillin, using tripalmitin as the standard (Postma & Stroes 1968).

Energy losses and gains by each body component during gonad maturation and spawning were estimated using the following energy conversion factors: carbohydrate = 17.2 kJ g⁻¹, protein = 20.1 kJ g⁻¹, lipid = 35.3 kJ g⁻¹ (Brody 1945 and Beukema & De Bruin 1979). These factors were applied to mean values of each biochemical component measured during the reproductive cycle.

We present the tissue mass and biochemical components on a per animal basis, (ie, as absolute composition). This was possible as we sampled animals over a narrow size range. Other forms of presentation may confound interpretation of results (Couturier & Newkirk 1991) (ie, relative composition involves reciprocal relationships between the components and this may lead to misinterpretation of the underlying biologic phenomena).

Statistical Analyses

For all comparisons between sampling dates for each sex we analyzed the data using one-way ANOVAs to test the null hypotheses of no differences between reproductive stages (Sokal & Rohlf 1981). Comparisons between sexes at specific sampling dates used *t*-tests. Normality was tested using a Shapiro-Wilk's test (SAS 1991) and homogeneity of variances using a Levene test (Snedecor & Cochran 1989). LS means multiple pairwise comparisons were used to test *a posteriori* for specific differences when the ANOVAs indicated significant ($P \leq 0.05$) differences (SAS 1991).

RESULTS

Masses of Body Components

During the period from May 30 to August 14, 2000, marked changes in the wet and dry mass of gonad and somatic components were apparent, particularly in male scallops (Fig. 1). These changes occurred despite a lack of difference in shell height of the males sampled on these dates ($P = 0.68$). The wet and dry mass of the testes and ovary increased markedly between May 30 and July 20 and decreased precipitously by August 14, indicating gonad maturation and spawning, respectively. Testis dry mass was significantly ($P = 0.018$) larger than ovarian dry mass on May 30. This suggests that gonad maturation was advanced in males relative to females. Such a pattern was observed for this population of *C. islandica* during the reproductive seasons of 1990 and 1991 (D. Arsenault, personal communication). At maturity, the wet mass of the ovaries was greater than that of the testes ($P = 0.0005$), although dry masses were similar ($P = 0.53$), suggesting that ovaries are more hydrated when fully mature. In females, during maturation and spawning most body components (wet and dry masses) remained stable ($P > 0.05$), with the exception that the dry mass of the remaining tissues (mainly mantle and gills) was lowest after maturation and increased after spawning ($P < 0.05$) (see Fig. 1). In males, during gonad maturation and spawning, the digestive gland mass decreased steadily, attaining its lowest value after spawning ($P < 0.05$). Adductor muscle dry mass dropped ($P < 0.05$) during testis maturation and recovered slightly after spawn-

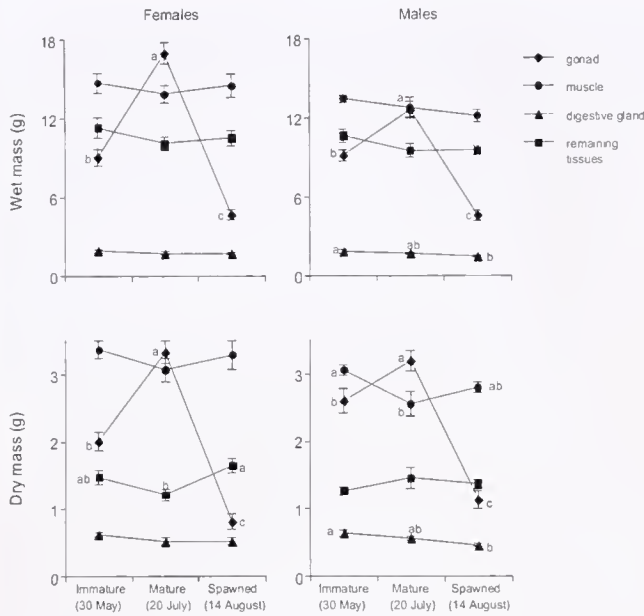


Figure 1. Total wet and dry masses (g) of the gonad, muscle, digestive gland and remaining tissues (mainly mantle) of females and males *C. islandica*, sampled at three different dates (May 30, July 20, and August 14, 2000) corresponding to when gonads were immature, mature and spawned. Values represent means \pm SE ($n = 15$). Means sharing the same letters were not significantly different ($P < 0.05$) as determined by LS means multiple comparisons.

ing, whereas the mass of the remaining tissues stayed constant (see Fig. 1).

No sex differences in the mass of the adductor muscle, digestive gland, and remaining tissues were observed ($P > 0.05$) before and after gonad maturation. However, after spawning, the wet and dry masses of the adductor muscle, digestive gland, and remaining tissues were lower ($P < 0.05$) in males than in females. This difference was not significant for the digestive gland when measured as dry mass ($P = 0.059$).

Biochemical Components

During gonad maturation, the absolute contents of carbohydrate and protein in ovary and testis increased markedly. Lipid levels only increased in the ovary (Fig. 2). In parallel, glycogen in the adductor muscle decreased by 160 and 185 mg in females and males, respectively, carbohydrates in the remaining tissues dropped by 70 and 66 mg in females and males respectively, and male scallops showed a 15 mg decrease of digestive gland carbohydrates. In contrast, the carbohydrate content of the female digestive gland increased by 19 mg during maturation (see Fig. 2). During gonad maturation, protein contents remained constant in all somatic tissues, and somatic lipids only decreased in the male digestive gland (467 mg) (see Fig. 2).

After spawning, all biochemical components of the ovary and testis dropped to their lowest levels (see Fig. 2). In female and male scallops, muscle carbohydrate remained low, however carbohydrate in the remaining tissues returned to pre-maturation levels. Protein levels in the digestive gland decreased by 80 mg with spawning of male scallops, whereas lipid levels decreased by 288 and 400 mg in females and males, respectively (see Fig. 2).

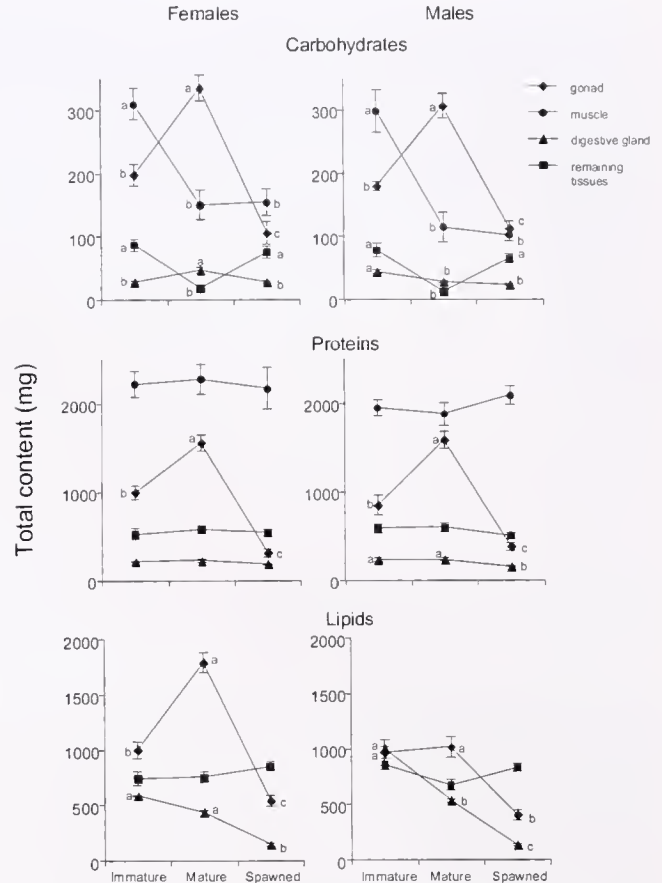


Figure 2. Total content (mg) of carbohydrates, proteins and lipids in the different body components (gonad, muscle, digestive gland and remaining tissues) of females and males *C. islandica*, when they were immature, mature and spawned. Values represent means \pm SE ($n = 15$). Means sharing the same letters were not significantly different ($P < 0.05$) as determined by LS means multiple comparisons.

Energetic Changes During Maturation

During the period of ovary maturation we studied, female somatic components lost a total of 4 kJ (taking only statistically significant variations into account), while the ovary gained 41.5 kJ (Table 1). Thus, somatic tissues contributed only 10% of the energetic gain of ovaries. Adductor muscle provided 70% and the remaining tissues 30% of this energy. Only carbohydrate was responsible for the energetic loss of somatic tissues in females. If the decrease in lipids in the digestive gland, even though it was not significant, is included in this calculation, 25% of the ovarian energy gain was explained.

During the period of testis maturation we studied, somatic tissues lost a total of 21 kJ, while testis gained 17 kJ (Table 2). Therefore, the energetic gain of the testis was completely supported by somatic tissues. Digestive gland accounted for 80%, adductor muscle 15%, and the remaining tissues 5% of the total caloric loss in the somatic tissues during testes maturation. In the digestive gland 99% of the energetic reduction was caused by lipid loss and 1% to carbohydrate loss. The total caloric loss in the adductor muscle and remaining tissues was caused by carbohydrate. The remaining tissues also showed a marked reduction of lipid but, as in the digestive gland, this was not statistically significant.

TABLE 1.

Caloric (kJ) content in the biochemical components of tissues of female *Chlamys islandica*, in different reproductive stages, and significant ($P < 0.05$) caloric losses and gains (ΔE) after gonad maturation and spawning.

Reproductive Stage	Protein	ΔE	Carbohydrate Muscle	ΔE	Lipid	ΔE	Σ Substrate	$\Sigma \Delta E$	ΣE Losses
Immature	44.6		5.3		n.e.		49.9		
Mature	45.7	1.2 (n.s.)	2.6	-2.8	n.e.		48.3	-2.8	-2.8
Spawnd	43.6	-2.1 (n.s.)	2.7	0.1 (n.s.)	n.e.		46.3	0	0
Digestive Gland									
Immature	4.3		0.5		20.7		25.5		
Mature	4.5	0.2 (n.s.)	0.8	0.3	15.3	-5.4 (n.s.)	20.6	0.3	0
Spawnd	3.7	-0.8 (n.s.)	0.5	-0.3	5.1	-10.2	9.3	-10.5	-10.5
Remaining Tissues									
Immature	10.6		1.5		26.2		38.3		
Mature	11.6	1.0 (n.s.)	0.3	-1.2	26.7	0.5 (n.s.)	38.6	0.3	-1.2
Spawnd	10.9	-0.8 (n.s.)	1.3	1.0	30.2	3.5 (n.s.)	42.3	1.0	0
Gonad									
Immature	20.1		3.4		35.3		58.7		
Mature	31.3	11.2	5.8	2.4	63.2	27.9	100.3	41.5	0
Spawnd	6.3	-25.0	1.8	-4.0	19.2	-44.0	27.3	-73.0	-73.0

(n.s. = not significantly different, $P < 0.05$, n.e. = not evaluated)

During the period of gonad maturation we studied, somatic proteins made no energetic contribution in males or females. In males, lipids represented 78% and carbohydrates 22% of somatic energy loss. In females, only carbohydrate reserves showed statistically significant changes. Although the energetic gain in the ovary was more than 2-fold that of the testis, the increments in protein and carbohydrate were similar in both gonads (see Tables 1 and 2). The difference in energy gain reflected the marked increase in ovarian lipids, whereas the change in testicular lipids was not significant.

Energetic Changes With Spawning

During spawning, the ovary lost 73 kJ and testis 49 kJ, confirming the greater energetic investment in egg production. The

remaining tissues in females and males increased their caloric content, mainly because of the recovery of initial carbohydrate levels (see Tables 1 and 2). Adductor muscle energy contents did not change. On the other hand, the energy content of the digestive gland dropped to its lowest level in females and males, mainly caused by lipid loss. Thus, the somatic contribution to energy requirements during spawning came from digestive gland lipids in female and male *C. islandica*. The total energetic decrease during spawning was 83.5 kJ in females and 65.2 kJ in males.

DISCUSSION

In the Mingan Islands, gonad maturation and spawning of *C. islandica* was accompanied by changes in body mass and biochemical components that differed markedly between males and

TABLE 2.

Caloric (kJ) content in the biochemical components of tissues of male *Chlamys islandica*, in different reproductive stages, and significant ($P < 0.05$) caloric losses and gains (ΔE) after gonad maturation and spawning.

Reproductive Stage	Protein	ΔE	Carbohydrate Muscle	ΔE	Lipid	ΔE	Σ Substrate	$\Sigma \Delta E$	ΣE Losses
Immature	39.2		5.1		n.e.		44.4		
Mature	37.8	-1.4 (n.s.)	2.0	-3.2	n.e.		39.7	-4.6	-4.6
Spawnd	42.0	4.2 (n.s.)	1.8	-0.2 (n.s.)	n.e.		43.7	4.0	-0.2
Digestive Gland									
Immature	4.7		0.7		35.3		40.8		
Mature	4.7	0.0	0.5	-0.3	18.8	-16.5	24.1	-16.7	-16.7
Spawnd	3.1	1.6	0.4	-0.1 (n.s.)	4.7	-14.2	8.2	-15.8	-15.8
Remaining Tissues									
Immature	11.8		1.3		30.3		43.5		
Mature	12.0	0.1 (n.s.)	0.2	-1.1	23.8	-6.6 (n.s.)	35.9	-7.6	-7.7
Spawnd	10.1	-1.8 (n.s.)	1.1	0.9	29.3	5.6 (n.s.)	40.6	4.6	-1.8
Gonad									
Immature	17.1		3.1		34.2		54.4		
Mature	31.9	14.8	5.3	2.2	35.8	1.6 (n.s.)	73.0	18.7	0
Spawnd	7.6	-24.3	1.9	-3.3	14.2	-21.6	23.7	-49.3	-49.3

(n.s. = not significant different, $P < 0.05$, n.e. = not evaluated)

females. Although spawning led to a considerably greater energy loss in female than in male gonads (73 vs. 49 kJ in gonad energy content), less mobilization of somatic energy was observed during gonad maturation and spawning in females than males (4 vs. 21 kJ during maturation and 10.5 vs. 15.7 kJ with spawning). The sex difference in investment during maturation (41.5 kJ vs. 17 kJ) was accentuated by the fact that the testes were larger than the ovaries at the start of our study, due to an advanced gametogenesis in males (Arsenault & Himmelman 1998). However, despite their greater reproductive investment, female scallops showed less somatic energy mobilization. Most of the energy requirements for ovarian maturation must have been covered by feeding, whereas somatic reserve mobilization could have covered the costs of maturation in males.

The sex differences in the changes of somatic reserves during gonad production and spawning were opposite those we predicted. What factors could explain the observation that female scallops can support the production of energetically expensive ovarian tissue by feeding, whereas males break down somatic tissues? Because the male and female scallops were sampled in the same habitat, food availability did not differ. If sperm production increases metabolic rate more than egg production, then the availability of aerobic power for food assimilation could be reduced thereby limiting feeding. Because the metabolic rate of mature male *C. islandica* is higher than that of mature females (Vahl & Sundet 1985), the aerobic scope available for feeding and assimilation could be lower in males than females. Protein levels increased more in the testes than in the ovaries during maturation, whereas lipids increased only in ovaries (see Fig. 2). *De novo* protein synthesis for sperm production is likely to be more costly than lipid deposition in the yolk. Protein synthesis requires activation of amino acids during their incorporation into nascent polypeptides and may require production of the appropriate amino acids. Because dietary fatty acids can be directly esterified into triglycerides and phospholipids, the energetic cost of lipid absorption and deposition is lower than the calorific value of the lipids. The close morphologic association between the digestive gland and the gonad could facilitate the direct transfer of lipids from food to gametes. Whereas these mechanistic explanations for the sex difference in breakdown of somatic reserves are speculative, our results suggest that male survival may be decreased by their reproductive investment whereas female scallops maintain greater survival and hence future reproduction.

In female *C. islandica*, the energetic gain by the ovary (41.5 kJ) during its growth was ~10 fold greater than the energetic loss in somatic tissues (muscle + digestive gland + "remaining tissues" = 4.0 kJ) (see Table 1). Even if the lipid change in the digestive gland (not significant) is taken into account, energy gain by the gonad was 4-fold the loss of somatic tissues. Therefore, because energy loss by somatic tissues must have accounted for only a small fraction of ovarian growth, external food supplies covered the remainder of this growth. The fact that, in *C. islandica* from the Mingan Islands, most ovarian maturation occurs during the spring-summer peaks of food concentration (Spence & Steven 1974, Arsenault & Himmelman 1998, and Brokordt et al. 2000a) suggests this is feasible. This is not what occurs in *Pecten maximus*, in which gonad growth takes place during the winter, and in which the energy loss by the adductor muscle was 23% larger than energy gain by the gonad (no differences were made between sexes because *P. maximus* is a hermaphrodite) (Faveris & Lubet 1991). Thus adductor muscle reserves in *P. maximus* support not only

gonad growth but also maintenance requirements, maybe because of low food availability during winter. These observations in *P. maximus* are similar to our results for male *C. islandica*. In summary, during gonad maturation, female *C. islandica* only mobilized carbohydrate reserves, mainly from muscle but also from the remaining tissues. Males mobilized both lipids (from the digestive gland) and carbohydrates (from all somatic tissues, but mainly from muscle), but lipid mobilization was more important in terms of amount (mg) and energy content (kJ). Therefore, the energetic support of ovarian maturation was carbohydrate based whereas testis maturation was primarily lipid based. During spawning, energetic demands in females and males are mainly supported by lipids from the digestive gland, but again males made a bigger somatic effort, losing 15.7 kJ versus a loss of 10.5 kJ in females (see Tables 1 and 2). Therefore, somatic energy loss during gonad growth and spawning was 2.5-fold greater in male than in female *C. islandica* (36.7 kJ and 14.5 kJ, respectively). Again this suggests that reproduction would reduce survival more in male than female scallops.

We analyzed the changes of biochemical components in the body tissues during gonad maturation and spawning in 13 species of scallops (Table 3) to compare with our results for *C. islandica* and to seek a common pattern for scallops. This compilation included species from different geographic zones and different degrees of biochemical analysis (not all of them included all somatic tissues or biochemical substrates). Most of the scallop species studied (11 of 13), used one or more energy reserves during gonad maturation. The two exceptions were *Chlamys septemradiata* from the Clyde Sea, England (Ansell 1974), and *Nodi Pecten subnodulosus* from Bahía Magdalena, Mexico (Racotta et al. 2001), species that increased biochemical components in all somatic tissues during gonad maturation. In these species, gonadal growth was supported directly and only by feeding. Other partial exceptions are some species or populations that have more than one reproduction or partial spawning per year, like *P. maximus* and *Aequi Pecten opercularis* from Ría de Arousa (Galicia, Spain) (Pazos et al. 1997 and Román et al. 2003) and *Chlamys varia* (Shafee 1981), in which spring gametogenesis and spawning are usually supported by external food supplies, which are abundant during this season. In contrast, autumn or winter reproduction in all the three species is supported by somatic tissue reserves. As a general pattern, and as found in female *C. islandica*, most species use carbohydrates (9 of 11), mainly from muscle and (8 of 9) as an energy substrate for supporting gonad maturation. Lipid and protein reserves were equally used during gonad growth (6 of 11), lipids coming mainly from the digestive gland (4), and proteins from adductor muscle (4). Reliance on muscle and mantle proteins is particularly pronounced in the semelparous *A. irradians concentricus* (Barber & Blake 1981) and *A. irradians irradians* (Epp et al. 1988). Thus, the literature shows that adductor muscle is the most important storage tissue providing energy substrates for gonad maturation in scallops, but that the extent of mobilization and the reserve used depends on food availability during maturation and the reproductive strategy (single vs. multiple spawners).

Of the 13 studies we analyzed, 10 evaluated the changes of biochemical components in body tissues during spawning (see Table 3). In some species, spawning is accompanied by recuperation of energy substrates depleted during gonad growth. This occurred in *Pecten maximus* from the Baie de Seine, France (Faveris & Lubet 1991), in *C. varia* from Bretagne, France (Shafee 1981), *Argopecten irradians irradians* from New York, United States

TABLE 3.

Changes of biochemical components in the body tissues during gonad maturation and spawning in thirteen species of scallops.

Species	Gonad Maturation	Spawning	Source
<i>Chlamys septemradiata</i>	↑ Muscle carbohydrate ↑ Mantle carbohydrate ↑ Muscle lipid ↑ Mantle lipid ↑ Muscle protein ↑ Mantle protein	↓ Muscle carbohydrate ↓ Mantle carbohydrate ↓ Muscle lipid ↓ Mantle lipid ↓ Muscle protein ↓ Mantle protein	Ansell 1974
<i>Chlamys islandica</i>			Brokordt et al. 2000 (present study)
Female	↓ Muscle carbohydrate ↓ Remaining tissues carbohydrate ↑ Digestive gland carbohydrate ↓ Muscle carbohydrate ↓ Remaining tissues carbohydrate ↓ Digestive gland carbohydrate ↓ Digestive gland lipid	↑ Remaining tissues carbohydrate ↓ Digestive gland carbohydrate ↓ Digestive gland lipid ↑ Remaining tissues carbohydrate ↓ Digestive gland carbohydrate ↓ Digestive gland lipid	
Male			
<i>Pecten maximus</i>	↓ Muscle carbohydrates ↓ Muscle lipid ↓ Muscle protein	↑ Muscle carbohydrate ↑ Muscle lipid ↑ Muscle protein	Faveris & Luhet 1991
<i>C. varia</i>	↓ Somatic carbohydrate ↓ Somatic lipid	↑ Somatic carbohydrate ↑ Somatic lipid	Shafee 1981
<i>Placopecten magellanicus</i>	↑ Muscle carbohydrate ↓ Muscle protein	↓ "Other tissues" protein	Couturier & Newkrk 1991
<i>Aequipecten opercularis</i>	↓ Muscle carbohydrate ↓ Muscle protein ↓ Digestive gland lipid		Taylor & Venn 1979, Román et al. 2001
<i>A. irradians irradians</i>	↓ Muscle lipid ↓ Muscle protein ↓ Mantle protein	↑ Muscle lipid ↑ Muscle protein	Epp et al. 1998
<i>A. irradians concentricus</i>	↓ Muscle carbohydrate ↓ Muscle protein ↓ Mantle protein ↓ Digestive gland lipid	↓ Muscle protein	Barber & Blake 1981
<i>A. ventricosus</i>	↓ Muscle carbohydrate ↑ Muscle protein ↑ Digestive gland protein		Chávez, unpubl. data
<i>Nodipecten subnodosus</i>	↑ Muscle carbohydrate ↑ Muscle protein ↑ Mantle carbohydrate ↑ Digestive gland carbohydrate ↑ Digestive gland lipid ↑ Digestive gland protein		Racotta et al. 2001
<i>Euvola ziczac</i>	↓ Muscle carbohydrate	↓ Muscle carbohydrate ↓ Muscle protein	Brokordt et al. 2000
<i>Lyropecten nodosus</i>	↓ Muscle carbohydrate ↑ Muscle lipid ↓ Remainder tissues carbohydrate ↑ Digestive gland lipid	↓ Muscle carbohydrate ↓ Remainder tissues protein ↓ Digestive gland lipid	Lodeiros et al. 2001
<i>Argopecten purpuratus</i>	↓ Muscle carbohydrate ↑ Muscle protein ↑ Muscle protein	↓ Muscle carbohydrate ↓ Muscle lipid ↓ Mantle carbohydrate ↓ Mantle lipid	Martínez 1991, Martínez et al. 2000

(Epp et al. 1988), and for mantle carbohydrates in male and female *C. islandica* (present study). However, most scallop species continue depleting the same biochemical reserves that were used during gonad maturation, or used reserves that were not used during gonad growth, to support energy demands during spawning. This was seen in *C. islandica*, *C. septemradiata*, *Placopecten magellanicus* from Nova Scotia, Canada (Couturier & Newkirk 1991), *A. irradians concentricus* from the Gulf of Mexico, United States (Barber & Blake 1981), *E. ziczac* from the Golfo de Cariaco,

Venezuela (Brokordt et al. 2000b), *A. purpuratus* from Coquimbo, Chile (Martínez 1991 and Martínez et al. 2000) and *Lyropecten (Nodipecten) nodosus* from the Golfo de Cariaco, Venezuela (Lodeiros et al. 2001). As during gonad maturation, adductor muscle reserves were most frequently used during spawning (5 of 10 species, Table 4). However, here muscle carbohydrates and proteins were of similar importance (4 and 3 species, respectively), followed by lipid reserves (4 of 10 species) from all three somatic tissues.

TABLE 4.

Proportion of scallop species ($n = 13$) using different biochemical components (and the primary source tissue) to support gonad maturation and spawning.

Reproductive Process	Carbohydrates	Proteins	Lipids
Gonad maturation	82% (88% muscle)	55% (66% muscle)	55% (70% digestive gland)
Spawning	50% (80% muscle)	50% (60% muscle)	40% (from all three tissues)

The reliance of scallops on the adductor muscle as an energy storage site has been suggested to reflect the monomyarian condition, which requires a readily available source of energy to support muscle contraction during swimming and escape responses (Ansell 1974). Muscle contraction is initially supported by ATP production from arginine phosphate, with octopine production from glycogen occurring in the final stages of exhaustive escape responses (Thompson et al. 1980, Livingstone et al. 1981, Chih & Ellington 1983, and Chih & Ellington 1986). The escape response and swimming performance would thus be largely maintained even after reduction of muscle glycogen levels. Recuperation from exhaustion relies on glycolytic breakdown of glycogen with ensuing octopine production (Thompson et al. 1980, Livingstone et al. 1981, Chih & Ellington 1983, and Chih & Ellington 1986). The preferential mobilization of carbohydrates for gonadal growth should primarily slow the capacity to renew escape responses. Effectively, the marked reduction of muscle glycogen during gonad maturation in *C. islandica* and *E. ziczac* is paralleled by decreases in muscle metabolic capacities and in recuperation from exhaustive exercise (Brokordt et al. 2000a and Brokordt et al. 2000b). On the other hand, in most scallops the adductor muscle contains 2–10-fold more protein than carbohydrate, suggesting that the mobilization of an energetically equivalent quantity of protein would cause a proportionally smaller impact on adductor muscle performance. However, several factors argue against the use of

protein. Muscle proteins have functional roles that make them an energetic reserve of last resort. Thus, mobilization of structural proteins (50%–60% of muscle proteins in *C. islandica* and *E. ziczac* (Brokordt et al. 2000a and Brokordt et al. 2000b)) would reduce muscle integrity. Even though soluble proteins are twice as abundant as muscle glycogen in *C. islandica* (Brokordt et al. 2000a), glycogen is used first, likely a reflection of the functional importance of these soluble proteins. Furthermore, the degradation of glycogen into sugar units and their use for the production of gonad carbohydrates or triglycerides would be metabolically simpler and energetically cheaper than the breakdown and resynthesis of proteins. Similar arguments underlie the use of lipids during ovocyte production. Explanations of the metabolic strategies underlying inter-tissue transfers of material and energy and reliance on dietary sources must integrate not only the energetic value of the components and the functional consequences of their use (ie, decreased behavioral responses) but also the energetic cost of their production.

ACKNOWLEDGMENTS

This study was supported by a grant from NSERC to HG. The authors thank Dominique Lapointe and Marie-Eve Fortin for careful technical assistance and to Martin Guay for the sampling of the scallops.

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ESTIMATION OF SEA SCALLOP ABUNDANCE USING A VIDEO SURVEY IN OFF-SHORE US WATERS

KEVIN D. E. STOKESBURY,* BRADLEY P. HARRIS, MICHAEL C. MARINO II AND
JACOB I. NOGUEIRA

School for Marine Science and Technology, University of Massachusetts Dartmouth, 706 South Rodney
French Boulevard, New Bedford, Massachusetts, 02744-1221

ABSTRACT A video survey was conducted from May 28, 2003, to August 23, 2003, to provide spatially explicit estimates of sea scallop density and size distributions along the off-shore northeast waters of the United States. Sea scallop (*Placopecten magellanicus*) densities in the Mid-Atlantic (26,270 km²) and Georges Bank (28,523 km²) ranged from 0.04 to 0.79 and 0.09 to 0.26 scallop · m⁻², respectively, and represented approximately 217,520 mt tons of scallop meats (approximately US\$2.4 billion). Sea scallops were highly aggregated in areas closed to mobile fishing gear. In the Georges Bank closed areas the proportion of sea scallop pre-recruits (<90 mm shell height) was low and sufficient to replace the adult population at an instantaneous mortality rate of 0.10 but not at a higher rate. A large number of prerecruit scallops were observed in the southern portion of the Hudson Canyon closed area extending south into open waters. Sea stars outnumbered sea scallops (approximately 39 to 16 billion, respectively) although most were small (20- to 40-mm arm length). Sea stars may be responsible for sea scallop mortality in the southern portion of Closed Area II.

KEY WORDS: sea scallop, *Placopecten magellanicus*, video, systematic survey, sea stars, Georges Bank, mid-Atlantic

INTRODUCTION

Large densities of sea scallops, *Placopecten magellanicus* (Gmelin 1791), exist in areas of the Mid-Atlantic and Georges Bank that have been closed to commercial scallop dredging since 1998 and 1994, respectively. Three areas on Georges Bank were closed to all mobile fishing gear to protect declining groundfish stocks, and two areas in the Mid-Atlantic were closed to enhance scallop abundance (Murawski et al. 2000, Hart 2001, 2003). To use the sea scallop resource in these areas, the New England Fisheries Management Council and National Marine Fisheries Service (NMFS) are developing fisheries management plans that require spatially explicit information.

Estimates of sea scallop abundance are made using dredge surveys, which require an estimate of dredge efficiency to determine absolute densities (Caddy 1989, Murawski et al. 2000). Using a video survey eliminates the error associated with the dredge efficiency as the actual number of scallops within the sample area is counted (Stokesbury 2002). Furthermore, this system provides spatially explicit information on the sea floor and marine benthic community (Stokesbury 2002).

The objective of this study was to provide spatially explicit, accurate, precise, absolute estimates of sea scallop density and size distributions along the off-shore northeast waters of the United States. Detailed information on the sediment and marine benthic habitat associated with the sea scallop fishing grounds will be presented in a future publication.

MATERIALS AND METHODS

In preparation for the survey Letters of Authorization for scientific research were obtained from the NMFS for each participating sea scallop-fishing vessel. Commercial fishing gear was removed from the fishing vessels prior to conducting each six-day cruise. A mobile studio, including monitors and S-VHS video recorders for each camera, a monitor for the Captain controlling the vessel's hydraulic winches to deploy the pyramid, a laptop

computer with Arcpad GIS® software integrated with a differential global positioning system with a WAAS receiver, and a laptop computer for data entry, was assembled in the wheelhouse. The survey grid was plotted prior to the cruise in Arcpad GIS®. Two scientists, a captain, mate and one deck hand were able to survey stations continually, completing 20 to 30 stations every 24 h.

The SMAST sampling pyramid was deployed from the scallop fishing vessels (Fig. 1). Two cameras mounted vertically on the pyramid at a height of 700 and 1575 mm above the pyramid's base provided quadrat sizes of 0.595 and 2.841 m², respectively (Figs. 2 and 3). A third camera mounted horizontally 50 mm above the pyramid base provided a side view across the sample area (Fig. 4). All sea scallops were counted, including those along the edge of the quadrat image that were only partially visible. To correct for this edge effect 75 mm, based on the average shell height of the scallops observed, was added to each edge of the quadrat image providing quadrat sizes of 0.788 and 3.235 m² (Stokesbury 2002).

Our primary quadrat sample size was 3.235 m², but one scallop per quadrat (0.31 scallop · m⁻²) is above minimal commercial densities (Caddy 1989, Brand 1991, Thouzeau et al. 1991, Stokesbury & Himmelman 1993, Stokesbury 2002). Therefore, at each station we collected four quadrat samples increasing the sample area to 12.94 m². The vessel stopped at each station and the pyramid was lowered to the sea floor. Footage of the first quadrat was recorded, and then the pyramid was raised so the sea floor could no longer be seen. The vessel drifted approximately 50 m and then the pyramid was lowered to the sea floor again to obtain a second quadrat; this was repeated four times.

We used a centric systematic design to position the stations because it is simple, samples evenly across the entire survey area, and has been successfully used to survey scallops on Georges Bank (Thouzeau et al. 1991, Stokesbury 2002). With this sampling design it is possible to estimate densities of macroinvertebrates within different areas without violating the sampling protocol or paying a statistical penalty for post-stratification. Further the centric systematic design facilitates mapping sea floor sediments and macroinvertebrate distributions.

Stations were positioned on a 5.6 by 5.6 km (3.0 by 3.0 nautical mile) grid overlying historical and present fishing grounds based

*Corresponding author. Phone: 508-910-6373; Fax: 508-999-8197;
E-mail: kstokesbury@umassd.edu



Figure 1. The SMAST video sampling pyramid aboard the F/V *Huntress*. The pyramid has a square base 2.2 m per side of 6 cm round iron, arms 2.5 m \times 4.5 cm round iron and weighs approximately 450 kg. It is deployed with the large hydraulic winch used in the scallop fishing industry while a second tension sensitive hydraulic winch controls the electronic cable. Rubber rings (three sets of eight rings, each 20 cm diameter, 5 cm thickness, per side) were placed on the base of this pyramid to prevent damage during deployment and provide gentle landings on the sea floor. Three underwater cameras (Deepsea Power & Light® multi-Seacam) and nine 100-w lights (Deepsea Power & Light® multi-Sealite) were attached to the pyramid.

on information from commercial fishermen and the Vessel Monitoring System (Rago et al. 2000; Fig. 5). We selected the distance of 5.6 km between stations because it was logistically feasible and allowed an accurate estimate of the mean sea scallop density with little loss of precision. Estimates of mean sea scallop $\cdot \text{m}^{-2}$ from the Nantucket Lightship area, surveyed in 2002, were similar for distances between stations ranging from 1.6 to 5.6 km; the standard error (SE) increased because of reduced sample size but the coefficients of variation (CV) were still low, 0.62 (SE = 0.057, CV = 9.3%) and 0.62 (SE = 0.101, CV = 16.3%) scallop $\cdot \text{m}^{-2}$, respectively. For distances greater than 5.6 km between stations the estimated means of scallop $\cdot \text{m}^{-2}$ as well as the standard errors and coefficients of variation increased, for example the mean was 0.75 scallop $\cdot \text{m}^{-2}$ (SE = 0.300, CV = 40.1%) for a 9.3-km station grid.

Video footage of the sea floor was recorded on S-VHS tapes. For each quadrat the time, depth, number of live and dead scallops, and latitude and longitude were recorded (Fig. 6). After each survey the videotapes were reviewed in the laboratory and an image of each quadrat was digitized and saved using Image Pro Plus®

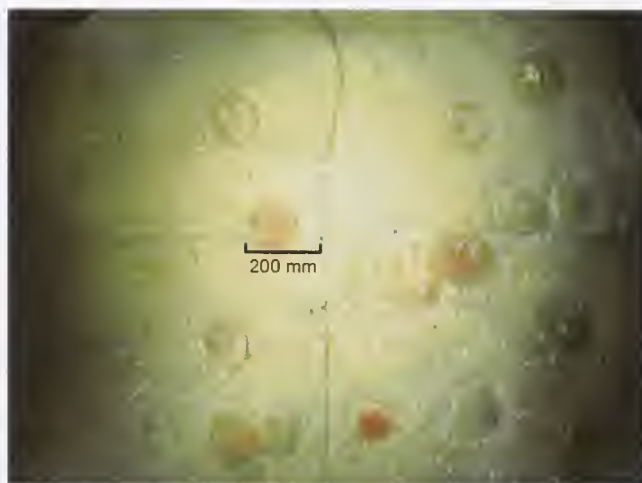


Figure 2. The 2.8-m² quadrat image digitized from video footage of sea scallops, *Placopecten magellanicus*, in the Nantucket Lightship Area (NLSA). Every quadrat image from this camera had a 200-mm mark clearly displayed on the lead-line cross.

software (TIF file format). Within each quadrat macroinvertebrates and fish were counted and the substrate was identified (Stokesbury 2002). Counts were standardized to individuals $\cdot \text{m}^{-2}$.

Sea scallop shell heights (mm) were measured using Image Pro Plus® software (Fig. 7). An equation (Equation 1) to correct for the curve of the camera lens was applied to each shell height measurement. This equation was estimated and verified with laboratory and field experiments that indicated a measuring error of less than 5% (author's unpublished data).

$$c = sh \sqrt{\frac{(x)^2 + (y)^2}{(y)^2}} \quad (1)$$

where:

c = corrected shell height (mm)

sh = shell height (mm) measured using image pro

y = vertical camera height from the base of the sampling pyramid

x = distance from the center of the quadrat (mm)

Mean densities and standard errors of scallops were calculated using equations for a two-stage sampling design (Cochran 1977).



Figure 3. The 0.6-m² quadrat image digitized from video footage of sea scallop, *Placopecten magellanicus*, prerecruits in the southern portion of the Hudson Canyon Closed Area.



Figure 4. Sessile macroinvertebrates, including the finger sponge, *Haliciona oculata* (Linnaeus 1759) and bryozoa, probably *Flustra foliacea* (Linnaeus 1738), digitized from video footage recorded with the side view camera. Note the pyramid frame and lights in the background.

The mean of the total sample is:

$$\bar{\bar{x}} = \sum_{i=1}^n \left(\frac{\bar{x}_i}{n} \right) \quad (2)$$

where:

n = primary sample units (stations)

\bar{x}_i = sample mean per element (quadrat) in primary unit i (stations)

$\bar{\bar{x}}$ = the mean over the two-stages.

The standard error of this mean is:

$$S.E.(\bar{\bar{x}}) = \sqrt{\frac{1}{n} (s^2)} \quad (3)$$

where:

$s^2 = \sum^n (\bar{x}_i - \bar{\bar{x}})^2 / (n - 1)$ = variance among primary unit (stations) means.

Because the sampling fractions were small, hundreds of scallops sampled compared with millions of scallops in the area, the finite population corrections were omitted simplifying the estimation of the standard error (Cochran 1977).

To examine the effect of variation between elements in the two-stage sampling design, sampling four quadrats per station, we created a simple formula in Excel (Microsoft, Seattle, WA) that randomly selected one of the four quadrats from each station and then calculated the means and variances for each survey area. This removed the subsampling component of the two-stage sampling design reducing it to an unaligned systematic sample, which is often superior to stratified random sampling (Cochran 1977). Using the Excel formula we generated 240 estimates of the means and determined the standard error (Glantz 2002).

The absolute number of scallops within a survey area was calculated by multiplying the mean number of scallops $\cdot m^{-2}$ by the total area surveyed (Stokesbury 2002). Distributions of scallops $\cdot m^{-2}$ were plotted using ArcGIS® software. Estimates of scallop meat weight (w) were derived from shell height (sh) frequencies for each area and length/weight regressions for each area to the nearest season and time of the survey. These equations were calculated from live dissections of sea scallops collected during

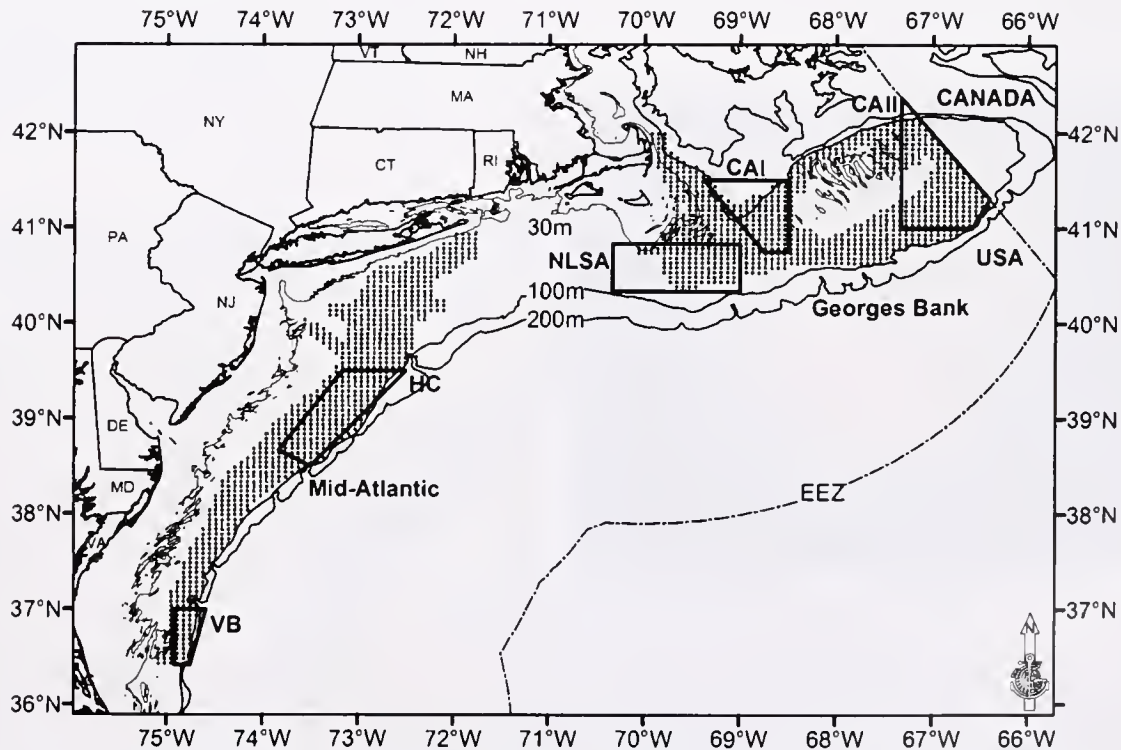


Figure 5. The SMAST 2003 video survey-sampling grid ranging from Virginia Beach, VA, to Cape Cod, MA, and to the Canada/US border (the hauge line) on Georges Bank. From May 28 to August 23, 2003, 1,775 stations, with four quadrats per station, were sampled on this 5.6 \times 5.6-km grid. NLSA, Closed Area I (CAI), Closed Area II (CAII), Hudson Canyon (HC), closed area, and Virginia Beach closed area (VB) are enclosed with a solid line; the Economic Exclusive Zone (EEZ) is also shown.

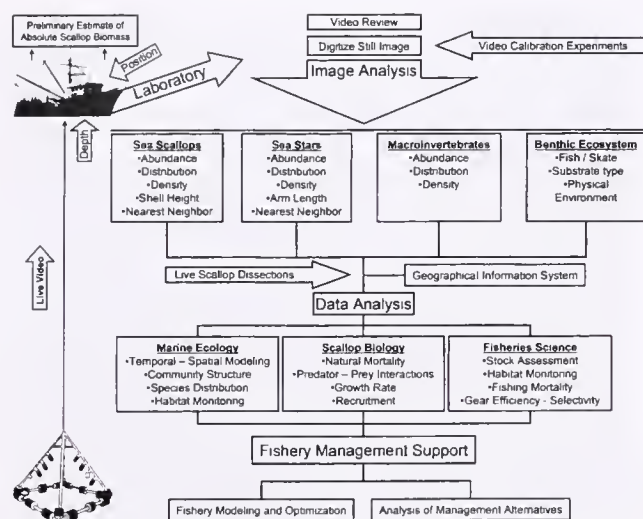


Figure 6. The SMAST video data flow chart detailing the procedures and analyses performed on the video footage collected during the 2003 survey.

fishing trips from open areas and from vessels that participated in the Sea Scallop Exemption Fishery in each of the closed areas (Table 2; Nantucket Lightship Area $w = 1.21 \times 10^{-5} \cdot sh^{3.1062}$, CAI $w = 8.21 \times 10^{-6} \cdot sh^{3.1321}$, CAII $w = 1.52 \times 10^{-5} \cdot sh^{3.0443}$, Georges Bank open $w = 7.38 \times 10^{-6} \cdot sh^{3.1559}$, mid-Atlantic open, HC and VA $w = 1.63 \times 10^{-5} \cdot sh^{2.9485}$; author's unpublished data).

RESULTS

The 2003 survey of commercial off-shore sea scallop fishing grounds covered 54,793 km² (16,000 nm²). Sea stars (Asteroidea)

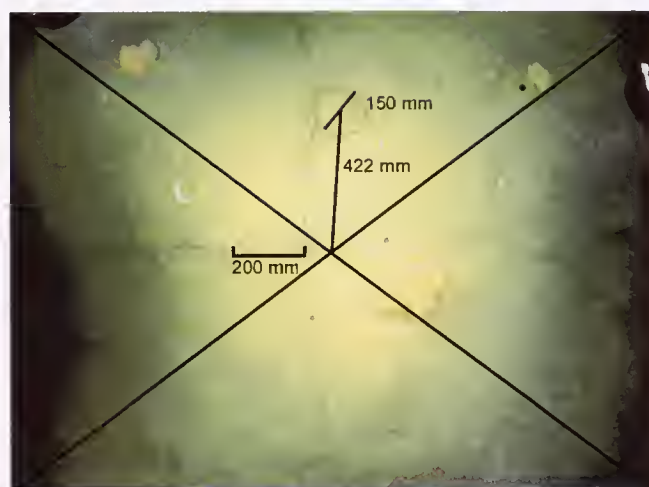


Figure 7. To measure the shell height (mm) of a sea scallop the digitized 2.8-m² quadrat image was imported into Image Pro Plus® software. Lines from each corner of the image were drawn to determine the center of the quadrat. The shell height was measured starting at the umbo to the shell margin. The scallop in this image is measured as 150 mm. The distance from the center of the scallop to the center of the quadrat was measured (422 mm, for this scallop). These measurements were entered into the correction equation, $150 \cdot \sqrt{(1572)^2 + (422)^2} / (1572)^2 = 155$ mm, which is the actual shell height of the scallop. Note the palmate sponge, probably *Isodictya palmate* (Lamark 1814) also in the quadrat and the sand ripple substrate.

were the most abundant macroinvertebrate observed numbering approximately 39 billion, although most individuals were small (20–40 mm arm length) (Table 1). High densities of sea stars occurred in both open and closed areas of Georges Bank and the mid-Atlantic with the highest density in the Nantucket Lightship area (Table 1).

Sea scallops, *Placopecten magellanicus*, were the second most abundant macroinvertebrate observed numbering approximately 16 billion scallops representing approximately 217,520 mt tons of scallop meats (Table 2).

The mid-Atlantic contained the greatest number of sea scallops observed in the 2003 survey, 74% (Table 2, Fig. 8). In the mid-Atlantic 23% of the area surveyed is closed to scallop fishing. The lowest and highest densities of scallops in the mid-Atlantic were in the Virginia Beach and Hudson Canyon closed areas, 0.04 and 0.79 scallop $\cdot m^{-2}$, respectively (Table 2, Fig. 9). Assuming that stations with at least one scallop observed in four quadrats represents scallop habitat (Stokesbury 2002) the densities increased to 0.09 (SE = 0.007) to 1.08 (SE = 0.338) scallop $\cdot m^{-2}$. Sea scallops were aggregated in the Hudson Canyon closed area, which contained 33% of the scallops observed in the mid-Atlantic, representing 36% of the scallop meat weight, (comparison of the number of scallops observed in open and closed areas, $df = 1$, $\chi^2 = 290$, $P < 0.001$).

The mid-Atlantic contained only 46% of the total scallop resource meat weight, (Georges Bank and mid-Atlantic combined; Table 2). This low meat weight compared with the high density of scallops is due to a large recruitment of scallops that were below commercially harvestable size (90-mm shell height equal to a 3.5" ring mesh size in the scallop dredge; Fig. 9). In the Hudson Canyon closed area and mid-Atlantic open area 62% and 69% of the scallops measured were less than 90 mm (Fig. 9).

Sea scallops were highly aggregated on Georges Bank with 66% of the all the individuals observed, representing 82% of the scallop meat weight, within the closed areas (comparison of the number of scallops observed in open and closed areas, $df = 1$,

TABLE 1.

The sea star, Asteroidea, density (sea star $\cdot m^{-2}$), number of stations sampled, standard error (SE), coefficient of variation (CV), and area surveyed (km²) using a two-stage sampling design.

Areas	Stations	Sea Stars			km ²	Millions of Sea Stars
		Mean m ²	SE	CV%		
Georges Bank						
NLSA	128	1.67	0.254	15.19	3951	262
CAI	97	0.09	0.023	26.76	2994	740
CAII	186	0.13	0.050	38.88	5742	5387
Open	513	0.34	0.032	9.30	15836	6618
Sum	924				28523	13007
Mid-Atlantic						
HC	160	0.97	0.131	13.47	4939	4793
VA	34	0.46	0.111	23.99	1050	487
Open	657	1.02	0.063	6.21	20281	20673
Sum	851				26270	25952
Total	1775				54793	38959

Estimates were calculated for the Nantucket Lightship Area (NLSA), Closed Area I (CAI), Closed Area II (CAII), Hudson Canyon (HC), and Virginia Beach (VB) closed areas and open areas of Georges Bank and the mid-Atlantic.

TABLE 2.

The sea scallop, *Placopecten magellanicus*, density (scallop \cdot m $^{-2}$), number of stations sampled, standard error (SE), coefficient of variation (CV), using a two-stage sampling design and an unaligned systematic sample.

Areas	Stations	Two-Stage Design			Unaligned Design			Meat Weight (g)	km ²	Millions of Scallops	Scallop Meats mt tons
		Mean m ²	SE	CV%	Mean m ²	SE	CV%				
Georges Bank											
NLSA	128	0.21	0.040	19.4	0.21	0.029	14.0	39.8	3951	816	32465
CAI	97	0.16	0.036	22.1	0.16	0.021	12.7	30.7	2994	489	15016
CAII	186	0.26	0.045	17.4	0.26	0.021	8.2	33.2	5742	1474	48980
Open	513	0.09	0.008	9.3	0.09	0.007	7.4	14.5	15836	1436	20823
Sum	924								28523	4215	117285
Mid-Atlantic											
HC	160	0.79	0.250	31.5	0.79	0.064	8.0	9.1	4939	3915	35714
VA	34	0.04	0.008	21.1	0.04	0.017	42.9	13.1	1050	41	533
Open	657	0.40	0.076	19.2	0.40	0.024	6.1	7.9	20281	8075	63988
Sum	851								26270	12030	100235
Total	1775								54793	16245	217520

Meat weight (g) estimates, based on the shell height frequency observed in each area and the shell height/meat weight regression, and area surveyed (km 2), provided the metric tons of meat weight in each area. Estimates were calculated for the Nantucket Lightship Area (NLSA), Closed Area I (CAI), Closed Area II (CAII), Hudson Canyon (HC) and Virginia Beach (VB) closed areas and open areas of Georges Bank and the mid-Atlantic.

$\chi^2 = 330$, $P < 0.001$; Table 2, Fig. 10). Densities of sea scallops ranged between 0.16 and 0.26 scallop \cdot m $^{-2}$ in the closed areas of Georges Bank compared with 0.09 scallop \cdot m $^{-2}$ in the open area (Table 2). Sea scallop densities increased to 0.38 (SE = 0.071) to 0.62 (SE = 0.094) scallop \cdot m $^{-2}$ in the closed areas and 0.23 (SE = 0.0175) scallop \cdot m $^{-2}$ in the open area for stations where at least one scallop was observed within four quadrats.

The majority of scallops in the open area of Georges Bank, 59%, were below harvestable size (<90 mm shell height; Fig. 9). The majority of sea scallops in the closed areas were very large with 77%, 87%, and 87% of the scallops greater than 90-mm shell height in Closed Area I, Closed Area II, and the Nantucket Lightship Area, respectively (Fig. 9).

The two-stage sampling technique provided the same means as the unaligned systematic sample (Table 2). In all areas the CV decreased using the unaligned systematic sample except for the Virginia Beach closed area where both the number of stations and number of scallops observed were small (Table 2).

Scallops that had died of natural causes were identifiable in the video footage as the two shells were still attached at the umbo. The highest number of dead sea scallops occurred in the southern portion of Closed Area II (Fig. 11). A dense aggregation of large sea stars (9.20 sea star \cdot m $^{-2}$, mean arm length 123.5 mm, SD = 35.39) was also observed in this area with the dead scallops suggesting a localized high natural mortality event (Fig. 12).

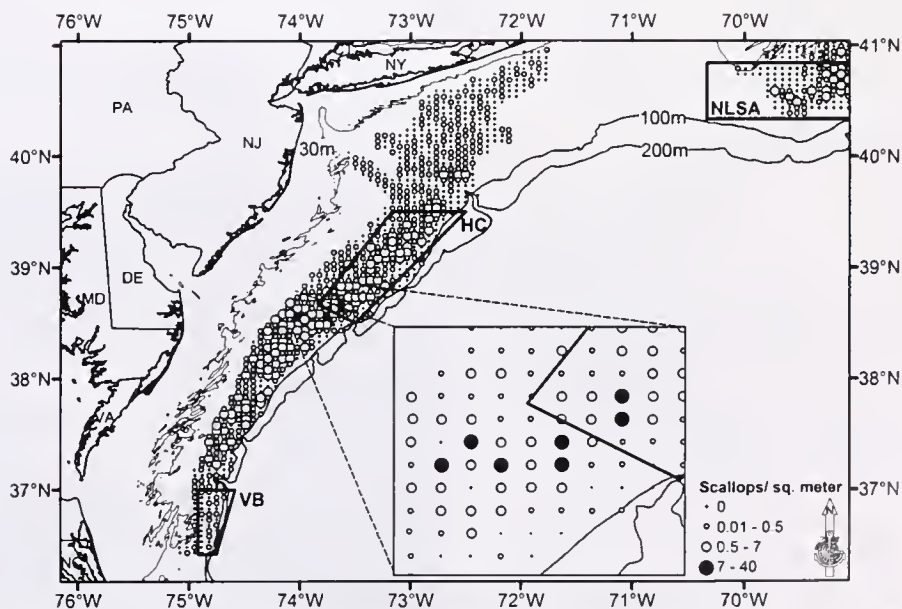


Figure 8. Sea scallop, *Placopecten magellanicus*, distributions (scallop \cdot m $^{-2}$) in the mid-Atlantic observed during the SMAST video survey. The insert shows the extremely high densities (>7 scallop \cdot m $^{-2}$) of pre-recruit scallops observed in the southern portion of the HC closed area extending south into the open area.

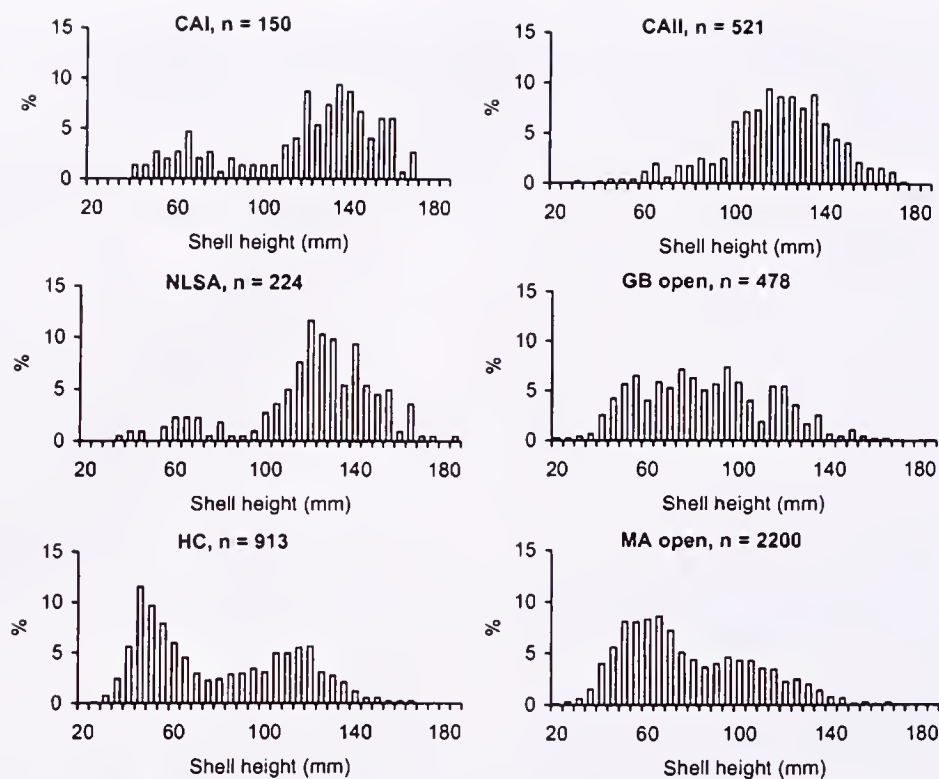


Figure 9. Shell height frequencies of sea scallops, *Placopecten magellanicus*, in the NLSA, CAI, and CAII, GB open, and in the HC closed area and Mid-Atlantic open area (MA open), n = number of scallops measured.

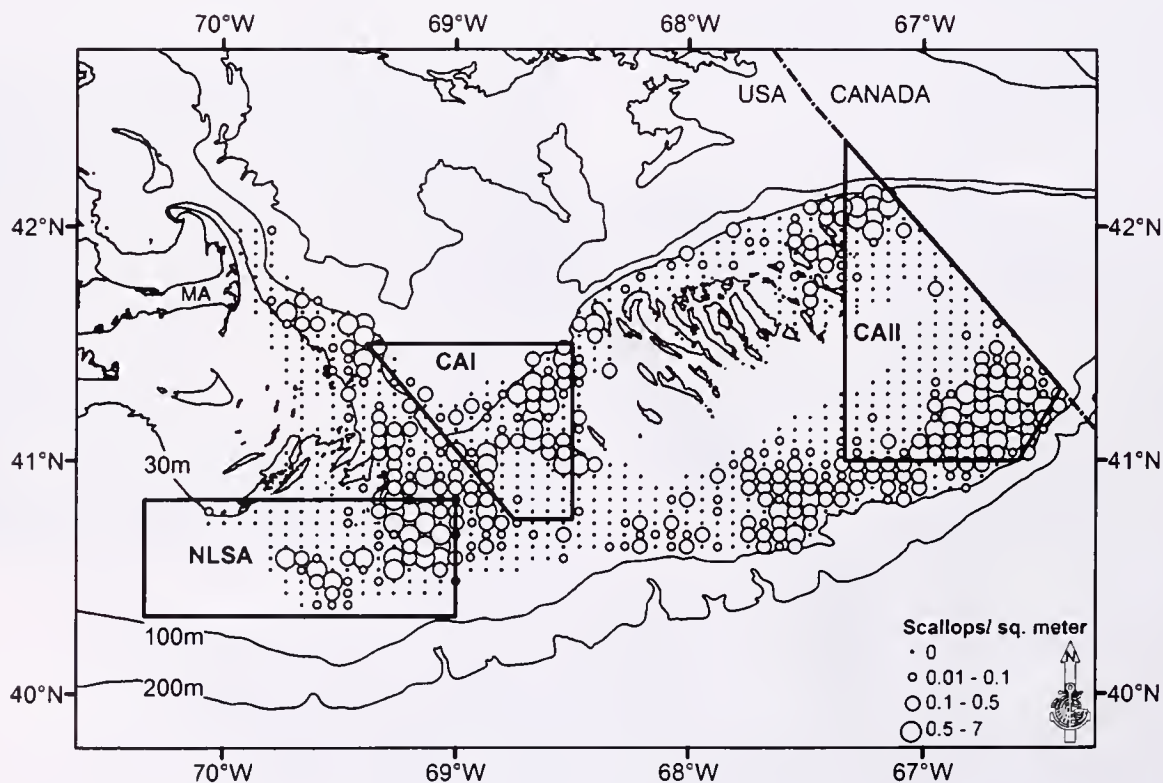


Figure 10. Sea scallop, *Placopecten magellanicus*, distributions (scallop · m⁻²) on Georges Bank in the open areas and in the NLSA, CAI, and CAII observed during the SMAST video survey.

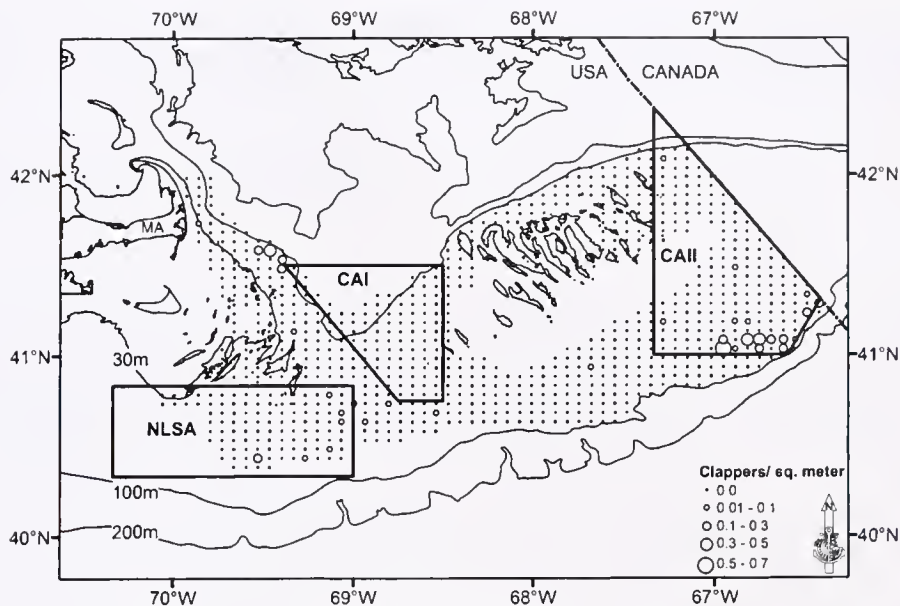


Figure 11. The distribution of dead sea scallops (clapper \cdot m⁻²), with the shells attached indicating natural mortality.

DISCUSSION

We estimate that the sea scallop, *Placopecten magellanicus*, resource in the off-shore waters of the Northeastern United States contain approximately 16 billion individuals, representing approximately 217,520 mt tons of scallop meats. The sea scallop biomass on Georges Bank in United States waters alone is 3.5 times greater than the average biomass from 1977 to 1988 for all of Georges Bank (31,500 mt tons, Canadian and United States portions combined; McGarvey et al. 1992). In the previous fishing year (March 2002 to February 2003) the sea scallop fishery harvested 23,237 mt tons of sea scallop meats, equivalent to 11% of the biomass surveyed in the 2003 survey (NMFS Fishery Statistics Report).

The high biomass is primarily a result of high densities and very large scallops within the closed areas of the mid-Atlantic and Georges Bank. However, sea scallops abundances have increased in the open areas as well.

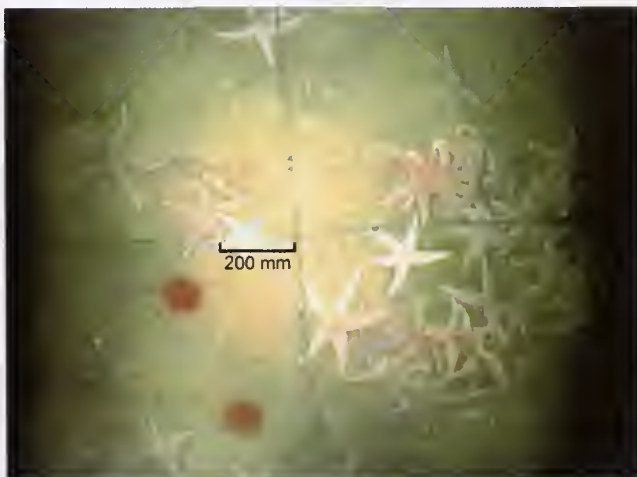


Figure 12. A high density of large Sea stars, *Asterias* spp., and two sea scallops observed in the southern portion of CAII June 27, 2003.

The increase in numbers of scallops in the mid-Atlantic is the result of an extremely large recruitment of juvenile scallops. Periodically large year classes of sea scallops have occurred throughout the history of the fishery and are believed to result from favorable environmental conditions (Serchuk et al. 1979, Caddy 1989). Caddy (1989) states that scallop biomasses have shown wide fluctuations from year to year that are not all the result of fishing. The sea scallop fishery falls outside the stable classification and represents a cyclical, irregular or spasmodic fisheries time series depending on the geographic location of the bed (Caddy & Gulland 1983, Caddy 1989).

The high abundance of large scallops within the closed areas may be producing many more larval scallops than in previous years. Sinclair et al. (1985), Caddy (1989) and McGarvey et al. (1992) emphasize the importance of larger size on the fecundity-per-recruit relationship. McGarvey et al. (1993) suggest that a stock-recruitment relationship exists for +5 y old sea scallops but that the fishery removes the majority of scallops greater than age 3.25 y. The sea scallop densities and sizes we observed exceed previously recorded densities and sizes over large geographic areas (Stokesbury 2002).

The high abundance of large scallops within the closed areas on Georges Bank may also be inhibiting recruitment. Few prerecruit scallops (<90-mm shell height) were observed in these closed areas. Replacement of the adult scallop stock is barely occurring in the Nantucket Lightship Area and Closed Area II with the prerecruit densities observed in 2003, using an instantaneous mortality rate of 0.10 and assuming a 17 y life span (Caddy 1989). If the instantaneous mortality rate is slightly higher than 0.10, for example 0.12, then the 2003 recruits would not sustain the adult scallop population at its present density.

A sea scallop mass-mortality event may be occurring in the southern portion of Closed Area II due to sea star predation. Here the highest densities of large sea stars and dead scallops occurred. The NMFS observed a high instantaneous mortality rate ($M = 0.6$) in this area although there is considerable uncertainty in the estimate due to the year-to-year variability of the survey (the mean

number of scallops per tow was 1255 in 2002 and 698 in 2003, >40 and >70 mm shell heights, respectively; D. Hart, personal communication). Between 1928 and 1961 nine mass mortalities occurred in the Gulf of St. Lawrence populations (Dickie & Medcof 1963). The more devastating widespread mass mortalities (up to 80% of the population) were attributed to water temperatures exceeding the upper lethal limits for scallops. The less severe mass mortalities (up to 25%) were attributed to sea star predation (Dickie & Medcof 1963).

Sea stars were twice as abundant as sea scallops in 2003, although many were very small suggesting a new recruitment. In our previous surveys of Georges Bank and the Mid-Atlantic sea scallops far outnumbered sea stars. Therefore, localized high natural mortality caused by sea star predation may be an increasing concern for the sea scallop fishery. We are examining predator-prey distributions and interactions and spatial variation in natural mortality.

The video survey technique is fast, accurate, precise, and provides information on the biology of scallops and the associated habitat without disturbing the sea floor (Stokesbury 2002). The two-stage sampling technique and the unaligned systematic sample provided similar means and low coefficients of variation compared with many invertebrate surveys (Krebs 1989).

The 2003 video survey was a success due to the strong collaboration and participation of the sea scallop fishing fleet and

supporting industries. Preliminary data from this survey have been presented to the New England Fisheries Management Council and the NMFS. A closure that will protect the area of high sea scallop recruitment in the southern portion of the Hudson Canyon closed area extending south into open waters has been proposed in the recent sea scallop fishery management plan. Further, the southern portion of Closed Area II may be opened earlier than scheduled due to the high natural mortality observed in this area.

ACKNOWLEDGMENTS

The authors thank B.J. Rothschild for his support and guidance. The authors thank the owners, captains, and crews who sailed with us on the *F/V Friendship*, *F/V Huntress*, *F/V Liberty*, and *F/V Nordic Pride*. M. Steinell (Deep Sea Light & Power), D. Bentley (Cortland Cable), D. Chambers (Maritime Hydraulics), and P. Clingman (Diversified Marine) helped us design and build the tension sensitive hydraulic winch, cables and sampling pyramid. P. Christopher (NMFS) and L. Gavlin (USCG) provided the Letters of Authorization and insured smooth transitions between fishing and surveying for the vessels. P. Rago (NMFS) and T. Truong provided helpful comments on sampling variance. M. Stokesbury and B. Rothschild reviewed and provided many helpful comments, which greatly improved the manuscript. Aid was provided by SMAST, the Massachusetts Department of Marine Fisheries, and the sea scallop fishery and supporting industries.

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SITE SELECTION FOR OYSTER HABITAT REHABILITATION IN THE VIRGINIA PORTION OF THE CHESAPEAKE BAY: A COMMENTARY

ROGER MANN* AND DAVID A. EVANS

Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062

ABSTRACT A significant body of knowledge has been generated during the past decade on disease tolerance of the native oyster *Crassostrea virginica*. A major opportunity to move into a large-scale field application phase of that knowledge has been presented by a 10-y commitment by the U.S. Army Corps of Engineers (ACOE) to a partnership in Virginia focused on widespread restoration of oyster resources for ecological purposes. The partnership involves ACOE, the Virginia Institute of Marine Science (VIMS), the Virginia Marine Resources Commission (VMRC), and the Chesapeake Bay Foundation (CBF). This collaboration will effect a sequenced restoration effort involving site selection, site restoration, brood stock addition from known genetic lines, evaluation of the stock in the new location for disease tolerance and/or contribution to cumulative recruitment, and, through adaptive management, will seek to optimize the widespread restoration of the oyster populations in Virginia. This contribution focuses on the importance of site selection in this effort, paying particular attention to the roles of (1) demographics and disease status on fecundity of brood stock, (2) larval feeding and growth rate in high-turbidity conditions typical of low-salinity sanctuaries from disease, (3) ontogenetic changes in larval behavior in such conditions, and (4) the role of estuarine circulation in retaining larvae in regions suitable for subsequent recruitment. We argue that while efforts to develop disease-tolerant brood stock may contribute to restoration efforts, without parallel guiding knowledge of items 1–4 above, efforts at restoration will at best be serendipitous, at worst be doomed to failure, and that site selection in restoration is crucial to success.

KEY WORDS: Chesapeake Bay, *Crassostrea virginica*, restoration

INTRODUCTION

A major opportunity to move into a large-scale field application phase of advances in disease tolerance of the native oyster *Crassostrea virginica* has been presented by a 10-y commitment by the U.S. Army Corps of Engineers (ACOE) to a partnership in Virginia focused on widespread restoration of oyster resources for ecological purposes. The partnership involves ACOE, the Virginia Institute of Marine Science (VIMS), the Virginia Marine Resources Commission (VMRC), and the Chesapeake Bay Foundation (CBF). This collaboration will effect a sequenced restoration effort involving site selection, site restoration, brood stock addition from known genetic lines, evaluation of the stock in the new location for disease tolerance and/or contribution to cumulative recruitment, and, through adaptive management, will seek to optimize the widespread restoration of the oyster populations in Virginia.

This contribution focuses on site selection, paying particular attention to the role of oyster demographics, fecundity, larval biology, and estuarine circulation in determining the probable success of long-term recruitment. A temporal context is important in developing the rationale for the proposed study. Oysters are a primitive molluscan form with an extensive fossil lineage. They use a primitive planktotrophic larval form. The complex life history of the species, the remarkable physiologic range of tolerance of the adult form and its individual longevity, have served it well in coastal regions that exhibit ephemeral (in geological time) appearance and disappearance of estuaries in dynamic coastal temperate climate regions. Larvae serve as the initial colonizing life stages as estuaries are formed—it is the behavior of these larvae in complex estuarine circulation that facilitates this initial invasion. The adult form establishes long-term residency of the estuary by providing a local larval source, ensuring continued recruitment when conditions allow, and accreting reefs to facilitate recruitment of subsequent generations. These are classic examples of source-

sink dynamics (Pulliam 1988, Pulliam et al. 1991, Pulliam et al. 1992, Hanski 1994) during periods of thousands of years. To persist, the adult form must survive the annual temporal variations in a local environment, whereas the larvae survive only a narrow window in that time frame. Oysters are a classic example of the evolution of two distinctly successful evolutionary life stages that are different in their individual environmental tolerances and optima. The complex life history and ancient lineage of oysters argue that the traits of the larval and adult forms are highly conserved [see contributions in McEdwards (1991), Hall & Wake (1999)]. Furthermore, within the conservative limitations of both life history stages, it is realistic to expect limited phenotypic plasticity in response to rapidly (on an evolutionary time scale) changing local environments. Indeed, in a classic evolutionary sense, it is rapid changes in local environments that lead to local extinction.

Consider the situation faced by a collaboration of scientists and managers in restoration of the Chesapeake Bay oyster resource. The watershed has been irretrievably altered in the short period since Colonial settlement with accompanying change in water quality in an absolute sense and in seasonal runoff variability. The estuarine environment has been and continues to be radically altered by fishing, shoreline development, and maintenance of navigable channels. Such rapid change in local conditions would be a stress contributing to local extinction. In addition, two diseases (*Perkinsus* and *Haplosporidium* = MSX) are now endemic in the local populations, one of which (MSX) did not co-evolve with the local oyster populations. Such rapid changes in disease stress could contribute to local extinction. Extant oyster populations are limited to low-salinity sanctuaries. We know that oysters survive over a remarkable salinity range, but we do not know the extent of the low-salinity stress as a suboptimal environment—the abundant oyster literature is remarkably devoid of good data on low-salinity responses because so much literature is devoted to response in optimal environments. The low-salinity sanctuaries are in closer proximity to increasingly turbid regions of the upper estuary that adversely affect optimal feeding in both life-history stages, arguably more so in the larval stage. The exile of reproductive adults

*Corresponding author. E-mail: rmann@vims.edu

and larvae to low-salinity, turbid regions would again be a stress contributing to local extinction. Given this litany of stresses, it is indeed surprising that oysters have not become locally extinct.

Placement of three-dimensional reef sanctuaries and enhanced shell plantings (two-dimensional extensive substrate enhancement) cannot have the consistent expectation of stimulating long-term cumulative recruitment when placement is based on geological footprints of reefs that successfully survived for millennia prior to the radical environmental changes that have occurred since Colonial settlement. The environments in which those reefs formed reflect the conservative evolutionary traits of the native oyster—they do not reflect the current, post-Colonial local environment. A recent comprehensive map-based illustration of restoration activity to date in Virginia waters is given by Berman et al. (2002). Though the placement of reefs to date has been guided by a cumulative commonsense approach to data on both long-term disease impact and a much longer term history of oyster productivity (e.g., Baylor 1894, Haven et al. 1981), it has resulted in highly variable temporal (interannual) and spatial success in recruitment [see Bartol & Mann (1997), Luckenbach et al. (1999), Mann (2000), Mann (2001)]. This should not be surprising to us in that the larval and early postlarval stages are challenged by conditions that, we argue, are commensurate with local extinction. If we are to be successful in restoration of oyster populations, we must understand the limitations of the larval forms within this new (to them) suite of adverse environmental variables.

ILLUSTRATING THE CHALLENGE NUMERICALLY, PART I: COMPONENTS OF THE CALCULATION

The relative importance of brood stock fecundity, larval growth and survival, and larval retention in contributing to the recruitment of a subsequent generation can be illustrated by a numerical approach to life cycle descriptions. Originally and elegantly described by Paulik (1973), this approach was adapted by Mann and Evans (1998) in estimation of oyster, *Crassostrea virginica*, standing stock, larval production, and advective loss in relation to observed recruitment in the James River, Virginia. The current illustration simplifies this approach using a virtual population to examine the effects of three parameters on recruitment in subsequent generations. These parameters are (1) varying egg production by varying age-specific mortality of the parent population as a proxy for disease impacts, (2) varying duration of larval period in response to suboptimal feeding conditions, and (3) varying loss to advection related to estuarine tidal exchange. To summarize and simplify Mann and Evans (1998), recruitment, R , to the 25-mm size class is estimated from larval supply thus:

$$R = (F_{\text{tot}} \times F_q \times F_s \times F_d \times F_f) \times (1 - \text{exch})^{2d} \times (1 - L_{\text{mort}})^d \times P_{\text{sub}} \times P_{\text{foul}} \times P_{\text{met}} \times (1 - J_{\text{mort}})^{dp}$$

F_{tot} is total egg production and is estimated from size-specific fecundity. It is a cumulative total for all individuals (F_{ind}) in all size classes and typically estimated from length:dry weight estimators. In the current illustration, all sizes below 40 mm are considered young of the year (spat) and do not contribute to spawning, and fecundity is estimated from relationships given in Thompson et al. (1996) and Mann and Evans (1998). F_q is a sex ratio modifier. Cox and Mann (1992) suggest parity in sex ratio. Given the

lack of other data, a single sex ratio modifier, F_q , with a value of 0.5 (50% female in all size classes) is used in this illustration. F_s ; F_{ind} and hence F_{tot} can be modified based on salinity (S) effects. Mann and Evans (1998) suggested the following estimators for F_s :

$$\text{if } S > 13.5, F_s = 1.0; \text{ if } S < 13.5, \text{ then } F_s = [(S - 8.0)/(13.5 - 8.0)] \times 1.0 = (S - 8.0)/5.5$$

F_d modifies fecundity for disease effects with values ranging from 1.0 to 0.0. In the current illustration, it varies from 1.0 to 0.75 (a 25% reduction based on disease impact). F_f describes a density-dependent multiplier for fertilization efficiency with values from 1.0 (100% fertilization) to 0.0 (no fertilization). It is based on Levitan (1991) where:

$$\log \% \text{ fertilization} = [0.72(\log OD) + 0.49] \text{ or,} \\ \% \text{ fertilization} = [0.49 \times OD^{0.72}]$$

where OD is oyster density in numbers m^{-2} . In the current illustration, it is rewritten thus:

$$F_f = 0.0049 \times OD^{0.72}$$

Production of larvae (strictly speaking embryos or fertilized eggs) m^{-2} is therefore estimated by $(F_{\text{tot}} \times F_q \times F_s \times F_d \times F_f)$ in units of larvae m^{-2} .

Mann and Evans (1998) estimated retention of the larvae within the James River during planktonic development using the three-dimensional flow model of Hamrick (Hamrick 1992a, Hamrick 1992b) to provide source and sink data at scales within the estuary. For the current illustration, a simple dilution function, $(1 - \text{exch})^{2d}$, is used that assumes uniform dispersal within the estuary and proportional loss on each tidal cycle; that is, larvae are assumed to be neutrally buoyant and exert passive swimming behavior in response to oriented stimuli. Thus, larval numbers decreased with days with the duration of planktonic development by the function where exch is proportional volumes exchanged on each tide. The value of exch varies in the current study between 0.1 and 0.2 (0.2 equals a 20% exchange per tidal cycle), and d is the duration of the larval development (= planktonic) period. The correction $2d$ is used with a simple assumption of two tidal exchanges per day. In the current study, d varies from an optimum of 21 days, based on values from Mann et al. (1994), Mann and Evans (1998), Bochenek et al. (2001), and Powell et al. (2002), to a suboptimal value of 25 days based on assumed reduction of feeding and hence growth in low-salinity and/or high-turbidity regions.

The function $(1 - L_{\text{mort}})^d$ estimates larval mortality in the water column. L_{mort} is the daily larval mortality rate [a proportional value between 1.0 (all died) and 0.0 (no mortality)]. Survival is $(1 - L_{\text{mort}})$ for a period of one day or $(1 - L_{\text{mort}})^d$ for a "d" day planktonic development period. For the current illustration, L_{mort} is set at 0.05, 0.06, 0.07, 0.1, and an extreme value of 0.25. The decreasing exponential relationship ensures a gradual decreasingly sensitive response to increasing values of d . Modification of the original number of larvae to account for dispersal loss and mortality provides an estimate of larvae surviving to immediate premetamorphic size. The transition to an attached benthic form requires successful location of substrate, that the substrate not be occluded by competing organisms, and that the larvae have sufficient energy reserves to complete the metamorphosis to a juvenile feeding form.

P_{sub} , a dimensionless modifier with a value between 1.0 and 0.0, describes the probability of finding suitable substrate. The time scale and availability of shell substrate is crucial to successful recruitment (Morales-Alamo & Mann 1990). Consider that a shell layer 1-cm thick covering 1 m² of bottom has a volume of 10 L. For the current illustration, a premise is adopted that a shell layer a minimum of 1-cm thick is required to offer a suitable substrate. P_{sub} is estimated thus:

If shell volume > 10 L m⁻², $P_{\text{sub}} = 1.0$

If shell volume < 10 L m⁻², $P_{\text{sub}} = 0.1 \times \text{shell volume (in L)}$

P_{foul} describes proportional occupation of the substrate by competing organisms and varies between 1.0 (no fouling) to 0.0 (complete preclusion of settlement). Rheinhardt and Mann (1990) suggest a value of $P_{\text{foul}} = 0.33$ based on field studies in the James River. For the current illustration, a constant value of 1.0 is used.

P_{met} describes the probability of successful completion of metamorphosis to the attached form on a 1.0 (all survive) to 0.0 (no survival) scale. For the current application, the value is set at 0.20.

Recruitment, R , to the benthos is therefore estimated from larval supply values by incorporating $(1 - \text{exch})^{2d}$, $(1 - L_{\text{mort}})^d$, P_{sub} , P_{foul} , and P_{met} thus:

$$R = [(F_{\text{tot}} \times F_q \times F_s \times F_d \times F_f) \times (1 - \text{exch})^{2d} \times (1 - L_{\text{mort}})^d \times P_{\text{sub}} \times P_{\text{foul}} \times P_{\text{met}}]$$

$(1 - J_{\text{mort}})^{\text{dp}}$ modifies this estimator for postsettlement mortality and growth rates, both of which are known to be size dependent (Roegner & Mann 1995). Mann and Evans (1998) describe daily juvenile mortality rate as J_{mort} (proportional with a value between 0.0 and 1.0). Survival is $(1 - J_{\text{mort}})^{\text{dp}}$, where dp is the number of days to grow to a defined size. Based on values of J_{mort} in Roegner and Mann (1995), Mann and Evans (1998) suggest a cumulative mortality to 8-mm length of 93% during a 28-day period, a calculated value for J_{mort} of 0.09. Thus, $(1 - J_{\text{mort}})^{\text{dp}}$ for the current study is set at 0.07 to 8 mm length. Above this, length of J_{mort} is lower and set at 0.05 for another 25 days until a size of 25 mm when the surviving individuals are considered recruits to the subsequent generation (Eggleston 1990). For the current illustration, $(1 - J_{\text{mort}})^{\text{dp}}$ incorporates two mortality rates with a cumulative mortality value for the premetamorphosis larvae to 25-mm size class, including a P_{met} value of 0.20 is 99.84%, or a proportional survival of 0.0016.

ILLUSTRATING THE CHALLENGE NUMERICALLY, PART 2: DEVELOPING A GROWTH AND AGE VERSUS LENGTH ESTIMATOR

There are surprisingly few studies of oyster growth rate in the field in the Chesapeake Bay that can be directly related to expected growth on the bottom in reef situations. There are no such prior studies in the upper James River. For the current application, we used data from a growth study using two populations of naturally settled oyster spat collected in the James River in 1992. The populations were collected from dredge hauls on separate days and were thus treated as replicates. Spat on shells were placed in plastic mesh cages on the bottom at Horsehead reef in the upper James River [see Haven & Whitcomb (1983), Berman et al. (2002)]. Approximately 200 oysters were contained in each of three cages. Population #1 was collected on 10/15/92 contained in two cages, population #2 was collected on 11/11/92 and contained in one

cage. Population #1 was contained in two rather than one tray because of the mass of shell to which the oysters were attached. Measurements of length were made at regular intervals for random samples of oysters from within each cage(s) for the period 10/15/92–7/27/93 for population #1, and for the period 11/11/92–1/28/93 for population #2. After these respective periods, all oysters were measured (Table 1). Data for population #1 was pooled from both cages to avoid pseudoreplication. At sampling events, data were also collected on water temperature and salinity. A description of growth over time is obtained from a plot of time versus the mean length (maximum linear dimension) of the oyster (Fig. 1). The spat on shell were from summer 1992 recruitment but of unknown absolute age, thus time is given in Figure 1 as days after 1/1/92. Oyster growth varies seasonally such that a classic Von Bertalanffy equation describing growth would mask this important seasonal fluctuation. Thus, a modified Von Bertalanffy plot with growth oscillation corresponding to seasonal change in growth rate was used. This takes the following form:

$$L_t = \text{Linf.} \times \{1 - e^{-k[(t - t_0) + A - B]}\}$$

where: $A = C \times \sin[2 \times \pi \times (t - t_s)] / (2 \times \pi)$.

and $B = C \times \sin[2 \times \pi \times (t_0 - t_s)] / (2 \times \pi)$

where L_t is the estimated length at time t , Linf is asymptotic length, set at 120 mm based on field observations, K is the growth constant, t_0 is age at which length is zero, C is the amplitude of the growth oscillation, and t_s is the starting point of the oscillation with respect to $t = 0$ (1/1/1992).

TABLE 1.
Observed growth of oyster populations at horsehead.

Date	Temp (C)	Population #1 mean		Population #2 mean	
		<i>n</i>	length (mm)	<i>n</i>	length (mm)
10/15/1992	19.8	196	15.8		
11/11/1992	13.8	202	16.7	100	15.4
12/9/1982	7.6	140	16.2	79	16.4
1/28/1993	7	201	16.7	98	15.3
3/31/1993	12.4			175	14.1
4/14/1993		211	16.2		
5/3/1993	18.5	210	16.6	120	17.3
6/2/1993	22	239	16.7	94	17.9
6/28/1993	27.2	212	20.2	77	21.1
7/27/1993	28.8	208	25.3	76	26.6
8/24/1993	28.1	251	28.1	75	30.7
10/25/1993	19	251	33.2	75	36.8
11/16/1993	14.8	251	33.8	72	37.3
12/13/1993	8	243	33.9	72	37.6
4/4/1994	14	243	33.6	72	37.0
5/9/1994	18.5	235	33.2	72	36.7
6/10/1994	24.2	232	33.9	71	37.4
7/11/1994	28.6	234	37.1	70	41.4
8/8/1994	25.8	234	39.3	69	42.8
9/13/1994	24.5	230	41.4	69	43.9
10/18/1994	18	230	44.3	68	47.2
11/15/1994	17	230	45.3	68	49.0
2/20/1995	5	227	45.8	67	49.0
4/18/1995	16.9	229	45.4	65	49.1
5/24/1995	23.1	228	46.2	65	49.7
7/14/1995	28.9	160	47.2	38	50.7
8/29/1995	26.4	131	51	32	52.7
9/27/1995	20.7	135	52.2	30	52.9
10/17/1995	10.5	122	56.5	28	54.7

Observed and estimated growth of oysters at Horsehead

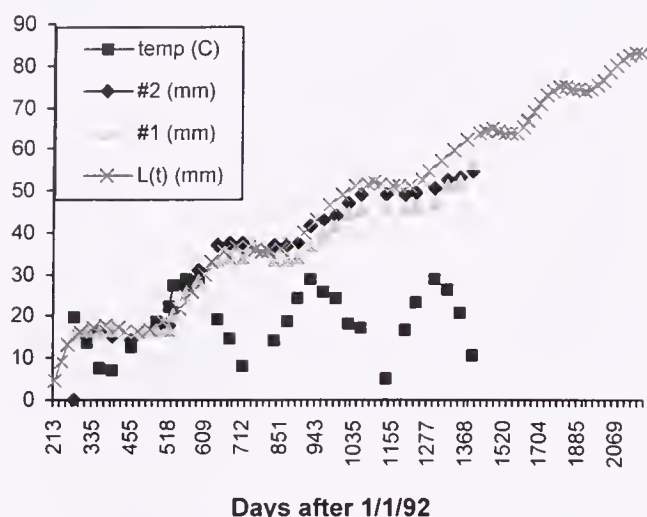


Figure 1. Observed and estimated oyster growth at Horsehead Reef, James River, 1992–1995.

The parameters of this function were estimated by fitting a rearranged function to the size increment data of a data set which records serial increase in length over time so that we have values of L_1 , L_2 , and so on. The rearranged function is:

$$L_2 = L_{inf} \times \{1 - (1 - L_1/L_{inf}) \times e^{-K[(t_2 - t_1) + A' - B']}\}$$

where $A' = C \cdot \sin[2 \times \pi \times (t_1 - t_s)/(2 \times \pi)]$,
and $B' = C \cdot \sin[2 \times \pi \times (t_2 - t_s)/(2 \times \pi)]$.

The parameters were estimated for this data set at the following values: $K = 0.204$, $t_0 = 0.36$, and $t_s = 0.608$. From length at age data, a matrix is created of both length and growth rate versus time. Estimated length, $L(t)$, versus time is superimposed on observed data in Figure 1. Estimated growth rate, expressed as mm/month increment, for each year class was calculated and subsequently rearranged as a rate versus temperature matrix. Matrix values were used to generate linear descriptors of monthly growth rate in relation to temperature for each year class. The relationship is in the form: $y = mx + c$ and is expressed as mm/month growth increments. Values are given in Table 2. There is a strong correlation between temperature and growth rate suggesting that the latter can be estimated from the former with a high degree of confidence, despite the obvious influence of seasonally varying factors, such as salinity turbidity and food supply, on growth.

Figure 1 illustrates a limitation of the seasonally oscillating fit equation: the possibility of negative growth rate estimation in the winter months. This is a product of the form of the equation with a positive and a negative component. The values used to generate the equation were means, and if error bars are generated around those means, then the brief period of negative growth inferred in Figure 1 is within the error bars. The negative mean growth rate values are small and are not further adjusted for the current model; however, the question arises of the most suitable form of an oscillating growth estimator, especially in a situation such as the James River where winter temperatures are sufficiently low to cause growth to cease, but the rapid spring rise in temperature results in a similarly rapid transition to a high growth rate. This rapid transition in growth rate may be easily masked in a typical

TABLE 2.

Estimation of size specific growth rate incorporating temperature effects.

Month	Temp (C)	Estimated Monthly Growth Rate (mm/month) = (mx + c) Where x is Mean Temperature		
			m	c
J	4.8			
F	5.1			
M	7.9			
A	13.7			
M	19.0	year 0	0.27	-2.80
J	24.1	year 1	0.20	-1.74
J	27.0	year 2	0.17	-1.42
A	27.2	year 3	0.14	-1.16
S	24.6	year 4	0.11	-0.94
O	19.2	year 5	0.09	-0.77
N	13.7	year 6	0.07	-0.64
D	8.3			

growth study with fixed time intervals. True representation of the transition in growth rate around the time of transition will require increased frequency of sampling.

A point of considerable impact that is illustrated by Figure 1 is the estimated age of James River oysters at 62.5 and 76 mm length, respectively (2.5 and 3.0 inches). Both lengths have been used to discriminate seed from market oysters in the commercial fishery in the decade of the 1990s. Though the popular consensus offered in public discussion of size limits in the James River public oyster fishery is that the difference in age between the two sizes is small, Figure 1 suggests otherwise. Animals may exceed the lower size limit in the age range 3.6–4.2 y, but the inflection of the length versus age curve in the mid-70s-mm range suggest that oysters of greater than 76 mm may be 5.5 or more years old. Thus, the increment from 62.5 to 76 mm length may require as much as two years to attain. The management implications are significant; decreasing the maximum size and subsequently reversing that size limit may require up to two years for stocks to recover to former demographics. Also, decreasing the size limit deprives the population of two extra year classes of spawning adults.

ILLUSTRATING THE PROBLEM NUMERICALLY, PART 3: AN EXAMPLE WITH A VIRTUAL POPULATION

Demographics for a virtual population were generated from a data set describing Horse Head Reef in the upper James River for the period 1994–1998 (R. Mann & J. Wesson, unpublished data shown here as Figs. 2A and 2B). This population was chosen because it was (a) stable over that period with respect to recruitment, total oyster density, and oyster demographics, and (b) suffered essentially no mortality due to disease. The size frequency distribution (in 5-mm size classes) was converted to an age frequency demographic using the age-length estimator described earlier.

The virtual population demography is illustrated in Figure 2C, as a series of populations (A–E inclusive) generated by gradually increasing age-specific mortality (illustrated as cumulative mortality in Fig. 2D) chosen to simulate the effects of increasing disease prevalence and intensity. It is notable that the extreme population, E, represents an approximation of current disease tolerance in the most selected strains under typical disease challenge in medium-

salinity waters. Each virtual population has the 25-mm size class, here considered the young of the year recruits or zero class, set at 100 oyster m^{-2} . This corresponds to the R value for recruitment to the benthos in the previously described estimator. In all simulations, performed as a sequential spreadsheet in Microsoft Excel, the barometer for maintenance recruitment of a subsequent generation is attaining a 25-mm size density of 100 oyster m^{-2} . The simulation was run for a single generation time frame with each of A–E as the starting demographic under various scenarios and the end points illustrated in Figures 2E–2L. Although these are just a subset of the many options that can be run with the simulations, they illustrate the following important points:

- (a) Under low tidal exchange ($exch = 0.10$) and optimum larval development ($d = 21$), the recruitment values are very high even with L_{mort} rates (Fig. 2E). With low L_{mort} rates, population A exhibits values of R approaching two orders of magnitude above a maintenance recruitment. Consider, however, that the scenario uses many optimal

conditions including no reduction in fecundity attributable to salinity, no shell limitation to settlement, and only modest competition for substrate. This is very much an optimal scenario.

- (b) Increasing larval duration by only 4 days ($d = 25$) reduces recruitment considerably (Fig. 2F), but still at least an order of magnitude above maintenance for optimal demographic profiles.
- (c) Increasing tidal loss to 20% drives all recruitment values below the critical 100 m^{-2} even with everything else at optimum (Fig. 2E).
- (d) Reducing fecundity by 25% as a proxy for impact of disease and/or salinity has a proportional effect (Fig. 2F).
- (e) Reducing fecundity by 25% and increasing tidal loss to 15% provides options for all population structure from A through E to recruit at $<100 m^{-2}$ depending on larval mortality rate—even with all other factors optimized!

The “take home” message from these illustrations is the

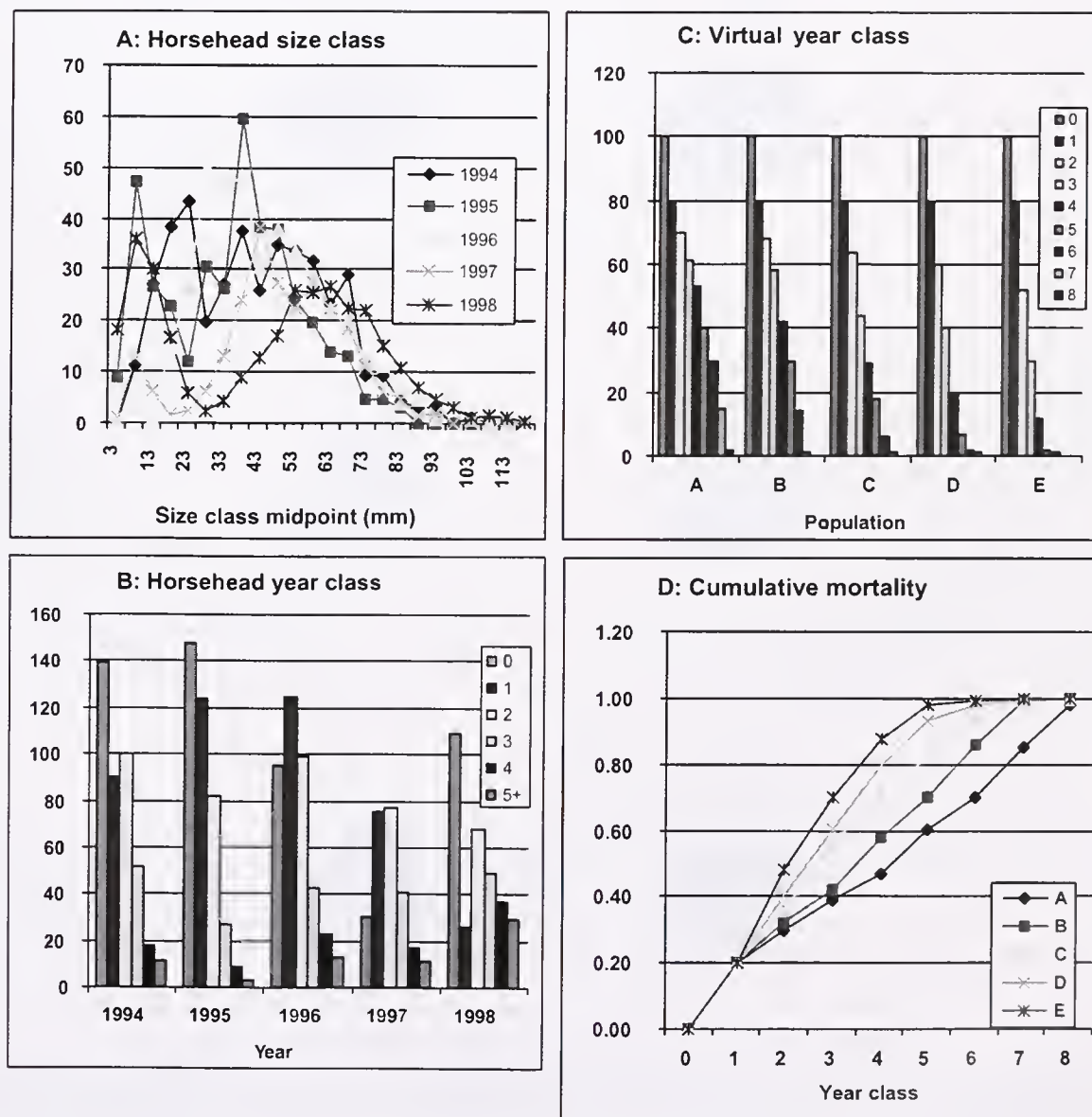


Figure 2. Continued on next page.

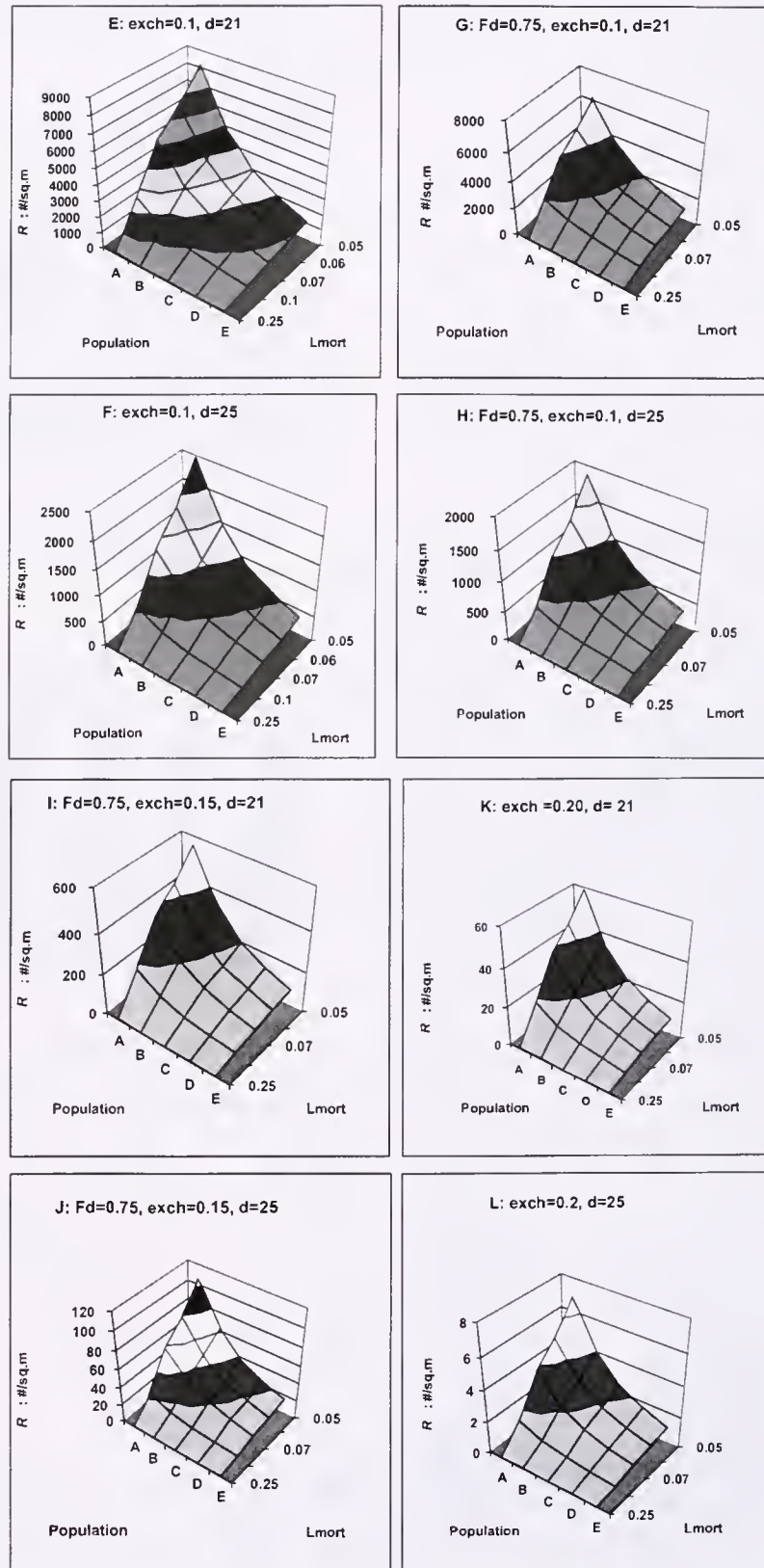


Figure 2. Estimation of recruitment, R , under various scenarios of initial population demographics, estuarine tidal exchange ($exch$), larval duration (d), and larval mortality rate (L_{mort}). See text for details. (A) Demographic data from Horsehead Reef 1994–1998. (B) Data recast as age class. (C) Virtual population demography used in simulations as a series of populations (A–E inclusive) generated by gradually increasing age-specific mortality (illustrated in D). (E–L) End points of simulations with varying values of $exch$, d , and L_{mort} .

very nonlinear response to various combinations of tidal exchange, reduced fecundity, and larval duration as we move away from an optimal combination. Even the most stable population structure, A in Figure 2C, may produce marginal recruitment scenarios despite the optimization of shell and substrate competition (P_{sub} and P_{foul}) and with no consideration of greater impact of postsettlement mortality. In a large number of slightly less than optimal scenarios, a population approaching current "disease tolerant" strains under sustained disease pressure—E in Figure 2C—is prone to inadequate recruitment. In other words, disease tolerance alone will not get us to where we want to be in current restoration work. In practical terms, failure to effect restoration in the optimal location will result in failure in recruitment. Optimal location is a product of species traits that are arguably very conservative because of the evolution of the species [again see McEdwards (1991), Hall & Wake (1999)] in combination with circulation patterns of the host estuary—a unique feature. Critical oyster traits in this mix include, but are not limited to, adult egg production, a trait for which we have not actively selected in breeding programs to date, and larval feeding ability and swimming behavior in turbid conditions. Fecundity is critical to driving the simulation as shown, yet we know essentially nothing of size–fecundity relationships under challenging conditions in which we are attempting restoration. Both larval traits are arguably very highly conserved because of structural limitations in the velar structure and the clear selective pressure over time for larval forms that recruit in optimal rather than sub-optimal environments. Turbid conditions can be viewed as transitions in the ephemeral lives of estuaries on a geological time frame, signals for oyster populations to move as they have done over periods of sea level rise. Larvae have no reason to evolve to survive in regions doomed to local extinction by rapidly changing environments—their conserved feeding abilities and behavioral strategies have served them more than adequately without such abilities. Restoration efforts thus match a suite of larval traits with conditions that we strongly suspect are very far from optimal, yet we often proceed in the absence of knowledge as to how debilitating this mismatch may be to the desired end point. These troubling scenarios, well founded in both our current understanding of the evolution of complex life history and simple numerical simulations of recruitment processes in virtual populations under near optimized conditions, are cause for concern. Without quantification of the individual data needs and their holistic synthesis in a practical model, the options for adaptive management of long-term, very high dollar cost restoration efforts, are limited, indeed sobering and probably doomed to mediocrity.

ARE DATA NEEDS FOR HOLISTIC SYSTEM LEVEL RESTORATION ACHIEVABLE?

The pressing need is to build a comprehensive model of oyster reproductive biology, larval growth and behavior, in response to estuarine circulation as a holistic adaptive management tool to guide restoration efforts for *Crassostrea virginica* in "low-salinity sanctuary" zones of the Chesapeake Bay. Fortunately, the tools for this are in place.

Disease-tolerant oyster strains have been, and continue to be developed under a multi-institutional, mid-Atlantic effort supported by the National Oceanic and Atmospheric Administration's Oyster Disease Research Program in a program whose heritage can be traced back to the pioneering efforts of Harold Haskin in Delaware Bay following the early impacts of MSX. Current hatchery

protocols allow for the description of the quantitative relationship between oyster size and fecundity at varying salinities typical of restoration sites. Whereas optimal salinity from literature studies may target a 15–25 ppt range, values in the 6–12 ppt range better reflect the upriver sanctuary regions of much of the Virginia sub-estuaries where extant oyster populations survive at the edge of endemic disease challenge. Culture of larvae from these fecundity studies at prevailing salinity under optimal and suboptimal (increased turbidity) conditions would greatly increase the confidence in growth and mortality rates as applied in the earlier simulation exercise.

The description of feeding under the combined stresses of low salinity and high turbidity remain poorly examined, although are eminently tractable in experimental systems. Mann, Kingsley-Smith, and Southworth (unpublished data) have used monocultured phytoplankton food and parallel cultures from the same source with additions of montmorillonite clay to simulate turbidity from upstream locations approaching the turbidity maximum; however, the challenge remains to use a complete characterization of low-salinity turbidity zones in terms of light penetration, particle concentration, and particle size in such experiments. The turbidity component of such data is emerging from separate studies of water quality on temporal and spatial variability in water column conditions in selected regions in the Virginia tributaries as these promote or limit submerged aquatic vegetation growth (Moore et al. 1996, Moore et al. 1997, Moore & Wetzel 2000). Sophisticated instrumentation for real-time, continuous generation of such data in transect mode is available. A critical issue yet to be examined is the changing quality of available food in these drainage conduits for disturbed watersheds. In such regions, increased run-off in conjunction with agricultural- and sewage-based nutrient enrichment serve to alter the balance of C:N:P:Si and thus the composition of the phytoplankton community. Concern over eutrophication typically focuses on mass rather than compositional issues, but it is inevitable that food quality will also change. Given the evolutionary history of larval forms, such changes can only be viewed as negative with concomitant prospects for recruitment to the benthos.

The contribution of oyster larval swimming behavior to larval retention has been extensively debated. Discussions of the additive, compounded, or antagonistic effects of these stimuli on larval swimming are offered in a series of papers by Mann (1985, 1986a, 1986b, 1988a, 1988b) and Baker and Mann (1997, 1998, 2003). Examination of swimming response to oriented stimuli are equally tractable in both laboratory and field settings using established protocols (Mann & Wolf 1983, Mann 1988a, McCarthy 1990, Mann et al. 1991, Baker & Mann 2003). The question in the current context is which (singular or plural) of these stimuli [light as intensity and/or wavelength, temperature, salinity (= density), pressure, and gravity] is relevant to the low-salinity location and is liable to modification by local increases in turbidity? Remember that we are seeking modification of a conserved behavioral response that has served the oyster larval form during the millennia, a modification particular to this recent (in geological time) temporal aberration from the optimum. In shallow upstream situations, we argue that the oriented responses to pressure are highly conserved [see the arguments for *Ostrea edulis* by Cragg & Gruffydd (1975)] and that stratification in both temperature and salinity will be minimal. This is substantiated for shallow locations in the James, Piankatank, and Great Wicomico Rivers from extensive summer survey data for the period 1985–2003 (reports available

on the VIMS Molluscan Ecology website at www.vims.edu/mollusc. We present the opinion that response to light in terms of both intensity and spectral composition as that most liable to modification, with resultant changes in larval depth stratification, and hence passive lateral dispersal; however, experimental verification of this stance must await future work.

The advancement of computer central processing power and code have fueled the development of three-dimensional transport models with biologically relevant cell sizes (with respect to known habitat heterogeneity) and time steps that have particle tracking capability in specific locations in the Chesapeake Bay. These models have been used in applications varying from water quality and sediment transport simulations to modeling circulation impacts of channel or shoreline alteration (such as in maintenance dredging or port construction), to modeling dispersal patterns of crab species with contrasting larval development (Garrison 1997), and critical placement of hard clam sanctuaries in the York River (see simulations at <http://www.vims.edu/physical/WEB/York1.htm>). All major restoration programs should have within their goals the development of such models as guidance tools.

Exploratory simulations can be run for virtual restoration scenarios driven by initial egg production estimates based on the modifications to the function ($F_{\text{tot}} \times F_q \times F_s \times F_d \times F_r$) as dictated by the projected additions of disease-tolerant broodstock. In a practical sense, we need realistic values for the function $(1 - \text{exch})^{2d}$ in various locations targeted for restoration in the Chesapeake Bay by the communal efforts of ACOE, VIMS, VMRC, and others. Historical observations on the role of the Piankatank and

Great Wicomico Rivers as trap-type estuaries (Andrews 1979) suggest this function to be small in both rivers. Indeed, both the Piankatank and Great Wicomico Rivers have successful histories of restoration activity (Southworth & Mann 1998, Luckenbach et al. 1999). The James River, the site of the only extant oyster fishery of any consequence in Virginia, is of historical context in terms of circulation (Pritchard 1953, Wood & Hargis 1971, Mann 1988a, Ruzecki & Hargis 1989) in that depth-related counter flows, gyre-like circulation in Hampton Roads, and tidally driven frontal systems all contribute to larval retention. These locations provide extensive historical data sets to blind test our simulations through hind casting. Iterative improvement of such simulations in turn provide for robust capability in forecasting mode and, ultimately, successful restoration of populations in the field. The challenge is simply to use this vast array of exciting tools in the task before us.

ACKNOWLEDGMENTS

This work was supported by NOAA Oyster Disease Research Program Grant No. NA26FL0385-01, NOAA Chesapeake Bay Stock Assessment Committee Grant No. NA66FU0487, and the NOAA Office of Sea Grant under Grant No. NA56RGO141. The assistance and discussions of our colleagues James A. Wesson, Melissa Southworth, Juliana M. Harding, Kenneth Moore, and Harry Wang are gratefully acknowledged. This manuscript is dedicated to our friends and colleagues Reinaldo Morales-Alamo and Kenneth S. Walker, both recently retired, in appreciation of their career contributions to the knowledge of oysters in the Chesapeake Bay.

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ECOSYSTEM INFLUENCES OF NATURAL AND CULTIVATED POPULATIONS OF SUSPENSION-FEEDING BIVALVE MOLLUSCS: A REVIEW

ROGER I. E. NEWELL*

*Horn Point Laboratory, University of Maryland Center for Environmental Science,
Cambridge, Maryland 21613*

ABSTRACT Suspension-feeding bivalves serve to couple pelagic and benthic processes because they filter suspended particles from the water column and the undigested remains, ejected as mucus-bound feces and pseudofeces, sink to the sediment surface. This biodeposition can be extremely important in regulating water column processes where bivalves are abundant in coastal waters and in seasons when water temperatures are warm enough to promote active feeding. Bivalves under these conditions can exert “top-down” grazer control on phytoplankton and in the process reduce turbidity, thereby increasing the amount of light reaching the sediment surface. This has the effect of reducing the dominance of phytoplankton production and extending the depth to which ecologically important benthic plants, such as seagrasses and benthic microalgae, can grow. Nitrogen and phosphorus, excreted by the bivalves and regenerated from their biodeposits, are recycled back to the water column and support further phytoplankton production. In some situations, however, bivalves can also exert “bottom-up” nutrient control on phytoplankton production by changing nutrient regeneration processes within the sediment. Some of the N and P that was originally incorporated in phytoplankton, but was not digested by the bivalves, can become buried in the accumulating sediments. Where biodeposits are incorporated in aerobic surficial sediments that overlay deeper anaerobic sediments, microbially mediated, coupled nitrification–denitrification can permanently remove N from the sediments as N_2 gas. Consequently, natural and aquaculture-reared stocks of bivalves are potentially a useful supplement to watershed management activities intended to reduce phytoplankton production by curbing anthropogenic N and P inputs to eutrophied aquatic systems. Environmental conditions at bivalve aquaculture sites should be carefully monitored, however, because biodeposition at very high bivalve densities may be so intense that the resulting microbial respiration reduces the oxygen content of the surrounding sediments. Reduction in sediment oxygen content can inhibit coupled nitrification–denitrification, cause P to become unbound and released to the water column, and the resulting buildup of H_2S can be toxic to the benthos.

KEY WORDS: benthic–pelagic coupling, bivalves, denitrification, eutrophication, extractive aquaculture, nutrient cycling, nutrient enrichment, nutrient trading, suspension-feeders

INTRODUCTION

Coastal waters worldwide are increasingly enriched with nitrogen and phosphorus as a consequence of agricultural fertilizer run-off and sewage inputs from growing human populations along coastal margins (Malone 1992, Conley 1999, Cloern 2001). This anthropogenic nutrient overenrichment is causing fundamental changes in the patterns and magnitude of primary production, including enhanced phytoplankton production and blooms of both toxic and nontoxic microalgae (Shumway 1990, Cloern 2001). In locations where this enhanced phytoplankton production exceeds the demands of metazoan herbivores, the excess phytoplankton carbon is metabolized by bacteria and protists and is not efficiently transferred to higher trophic levels (Pomeroy & Wiebe 1993). These high levels of microheterotrophic respiration frequently generate hypoxic or anoxic conditions in estuarine bottom waters. For example, in Chesapeake Bay, USA, water beneath the pycnocline in the deepest channel becomes anoxic most summers when the level of bacterial respiration, supported by the carbon produced during the spring phytoplankton bloom, exceeds the rate of oxygen resupply from the surface (Kemp & Boynton 1992, Diaz & Rosenberg 1995). In an effort to curb phytoplankton production in Chesapeake Bay, actions have been taken throughout the watershed to control inputs of nutrients from point and non-point sources, such that there will be an overall reduction by 40% of controllable inputs from the maximum levels recorded (D’Elia et al. 1992).

Phytoplankton concentrations increase not only as a result of

enhanced nutrient inputs—the so called “bottom-up” influence—but similarly increase when there is a reduction in the abundance of grazer organisms that normally exert “top-down” control (Newell 1988, Dame 1996). Thus, in eutrophic waters, consumption of particulate organic matter (POM) by abundant stocks of bivalve suspension-feeders in shallow and well-oxygenated conditions will directly reduce the amount of POM remineralized by bacteria beneath the pycnocline where oxygen resupply is restricted. Verwey (1952) was the first to identify the important ecological role of bivalves that are often the dominant component of the macroinvertebrate community in many coastal ecosystems. He recognized that bivalves serve as key agents in benthic–pelagic coupling because they feed on seston and transfer undigested organic and inorganic material in their feces and pseudofeces to the sediment surface.

The diverse influences that populations of suspension-feeding bivalves exert on marine ecosystem processes have comprehensively been reviewed by Dame (1996). I review here only those aspects of bivalve suspension-feeding ecology that pertains to their ability to alter water quality in eutrophic temperate estuaries and that may change the environment in a manner that affects other species. It is likely that some of these ecosystem effects will be a linear function of bivalve density, even at the rather high population densities associated with aquaculture farms, unchecked growth of an exotic species, and so forth. Other effects may be positive at low and moderate population densities but these beneficial changes can be reduced or lost at extremely high densities, as could result from overintensive aquaculture. I do not discuss the important secondary benefits of natural and cultivated shellfish beds as habitat for many invertebrate and vertebrate species (Coen et al. 1999). There is little known about such habitat benefits that

*Corresponding author. Fax: +1-410-221-8490; E-mail: newell@hpl.umces.edu

may derive from bivalves commercially cultured in various holding gear (e.g., rack and bag culture, attached to suspended ropes, held in floating trays, and so forth). These aquaculture structures are likely to provide a surface for fouling growth that serves as a food source for many animals and to some extent provide the type of spatially complex habitat that is sought by many species of animals. More research is required to determine to what extent the extreme periodic disturbances associated with cultivation and harvest practices, especially those required for on-bottom culture of infaunal bivalves, positively or negatively affect the habitat (Simenstad & Fresh 1995).

BIVALVE FEEDING

Suspension-feeding bivalves clear seston particles greater than $\sim 3\text{-}\mu\text{m}$ diameter from the water column with high efficiency during times of the year when water temperatures are sufficient to promote activity (Bayne & Newell 1983, Bayne & Hawkins 1992). Captured particles are sorted on pallial organs prior to ingestion, with the less nutritious and excess particles being immediately rejected as pseudofeces (Newell & Jordan 1983, Newell & Langdon 1996, Ward et al. 1997). In some situations, pseudofeces, which have not been exposed to any type of digestive degradation, can account for as much as 80% to 90% of the total volume of filtered particulate material (Tenore & Dunstan 1973, Bayne & Hawkins 1992).

The feeding response of bivalves to changes in seston concentration varies considerably among species. Some species, such as the eastern oyster, *Crassostrea virginica*, and the blue mussel, *Mytilus edulis*, maintain relatively high clearance rates even when seston concentrations increase (Newell & Langdon 1996, Hawkins et al. 1998). By maximizing the number of particles captured and subjected to efficient preingestive sorting and selection processes, such species can maximize their ingestion of nutritious particles (Newell & Langdon 1996, Ward et al. 1997). The maintenance of high feeding rates appears to be an adaptation to living in estuarine systems that historically supported low concentrations of phytoplankton in relation to less nutritious detrital and mineral particles. Today, throughout the majority of the estuaries and coastal waters of North America and western Europe, phytoplankton production has increased due to anthropogenic nutrient enrichment (Cloern 2001). Therefore, once the bivalve's nutritional needs are satisfied, excess phytoplankton cells, in addition to less nutritious detrital and silt particles, are rejected in pseudofeces. In response to increasing seston concentrations, other species of suspension-feeding bivalves, such as cockles, clams, and scallops, mainly regulate their ingestion rates by reducing clearance rates and not so much by rejecting excess particles as pseudofeces (Hawkins et al. 1998, Grizzle et al. 2001). Consequently, the species of bivalves that can exert the greatest influence on benthic-pelagic coupling are those that maintain high clearance rates and reject large numbers of particles as pseudofeces.

Ingested material is subject to extracellular and intracellular digestion, and the remains are defecated within ~ 24 h. Bivalves digest and assimilate different sources of POM with efficiencies that can vary from $\sim 20\%$ to 90% (Bayne & Newell 1983, Kreeger & Newell 2001). This efficiency varies depending on how susceptible the particles are to enzymatic breakdown; for example, many chlorophyte algal species are poorly digested because of their characteristically thick cellulosic cell walls (Langdon & Newell 1996). Assimilation efficiencies of bivalve molluscs for some particles,

including phytoplankton, also varies seasonally (Kreeger & Newell 2001). This high variability in assimilation efficiency, together with the substantial amounts of POM in pseudofeces that is not even subject to digestion, means that large amounts of undigested particulate organic nitrogen (PON) and phosphorus are transferred to the sediment surface in feces and pseudofeces (collectively called biodeposits). Newell & Jordan (1983), working with eastern oysters fed on natural seston ranging in concentration from 5 to 20 mg L^{-1} , reported that $\sim 50\%$ of the PON cleared from the water column was assimilated, and the remainder was voided in biodeposits.

Of the N absorbed by bivalves from the ingested food, the majority is used for tissue growth and some is excreted as urine (70% of which is NH_4^+ , 0% to 13% urea, and 5% to 21% amino-N; Bayne et al. 1976, Bayne & Hawkins 1992). This excreta increases the water column dissolved nitrogen pool and hence can support new phytoplankton and microphytobenthos production (Kaspar et al. 1985, Asmus & Asmus 1991, Swanberg 1991). Feces and pseudofeces are voided from bivalves as mucus-bound aggregates; consequently, they have a faster sinking velocity than nonaggregated particles and settle at rates up to 40 times that of nonaggregated particles (Kautsky & Evans 1987, Widdows et al. 1998). In locations where bottom water currents are below the critical erosional bottom shear stress (Newell et al. 2005), the biodeposits undergo a de-watering process and gradually become incorporated into the sediments (Haven & Morales-Alamo 1966, Haven & Morales-Alamo 1968, Kaspar et al. 1985, Jaramillo et al. 1992, Widdows et al. 1998) leading to an increase in sediment nitrogen content (Kaspar et al. 1985, Kautsky & Evans 1987, Deslous-Paoli et al. 1992, Hatcher et al. 1994). It should be noted, however, that resuspension of biodeposits from intertidal or shallow-water bivalve populations (Dame et al. 1991a) is more likely than those from bivalves living in either deeper water or grown in suspended aquaculture systems, where the underlying sediments are isolated from frequent disturbance by wave action.

ECOSYSTEM EFFECTS OF BIVALVE FEEDING

The complex relationships among some benthic and pelagic processes that may be influenced by benthic bivalve suspension-feeders during seasons when they are actively feeding are summarized in a conceptual model (Fig. 1). This diagram highlights the role of bivalve feeding in removing both phytoplankton and inorganic particles from the water column, thereby reducing turbidity. The resulting increased light penetration to the sediment surface can potentially enhance the production of benthic plants, such as seagrasses and microphytobenthos (Newell & Koch 2004). Reductions in turbidity will be directly proportional to the abundance of bivalves. Consequently, there is likely to be a linear decline in turbidity as bivalve stocks increase in a location from low to high abundances (Fig. 2).

A number of studies [for a review, see Dame (1996)] have provided strong evidence that natural populations of suspension-feeding bivalves can exert top-down control on phytoplankton in coastal waters [e.g., ribbed mussels, *Geukensia demissa* (Dillwyn), in salt marshes of the eastern United States (Jordan & Valiela 1982) and blue mussels, *Mytilus edulis* (Linné), in the North Sea (Asmus & Asmus 1991, Dame et al. 1991)]. Similar ecosystem changes have been observed where bivalve stocks have been either experimentally increased to higher than normal abundances [e.g., blue mussels stocked in experimental enclosures (Riemann et al.

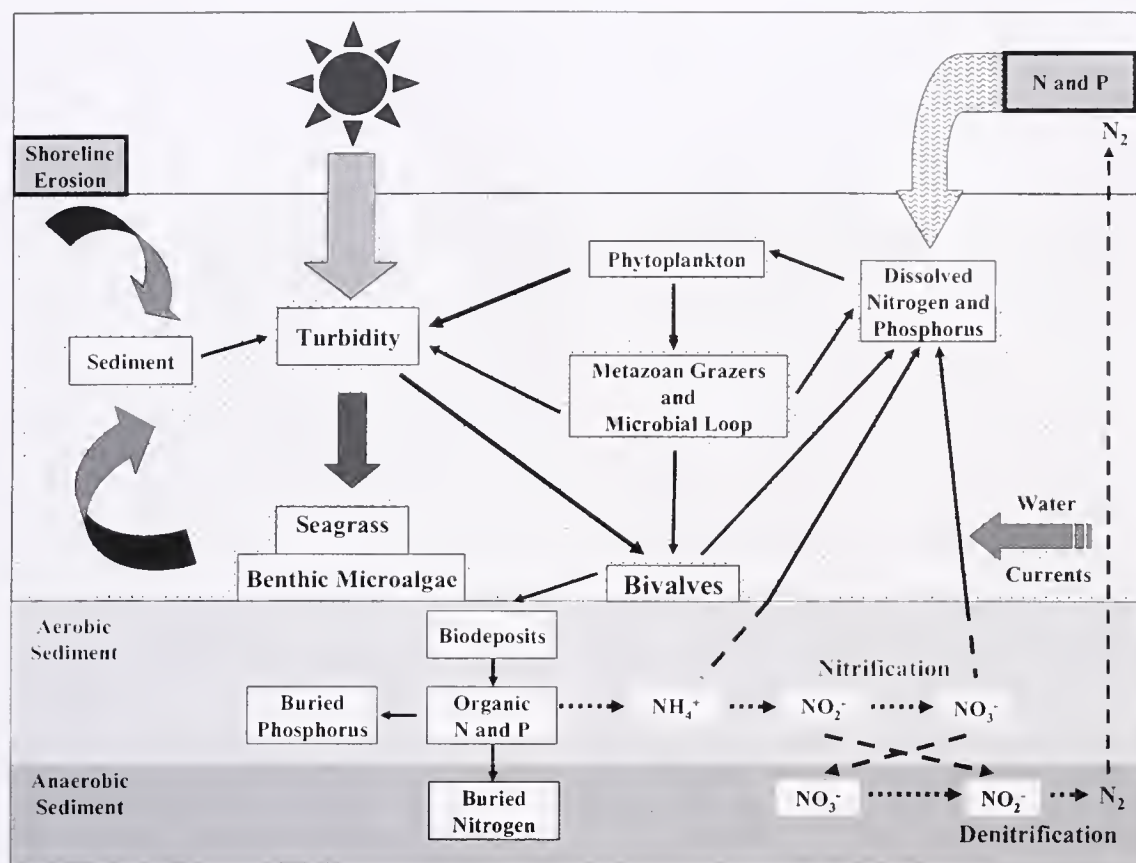


Figure 1. Conceptual diagram of the ecosystem effects of suspension-feeding bivalves in removing organic and inorganic particles from the water column and transferring undigested particulate material in their biodeposits to the sediment surface. Burial of P in aerobic sediments, and N throughout the sediment, leads to N and P removal from the water column. Within the aerobic sediment layers, the microbially mediated process of nitrification occurs, which when linked to denitrification within the underlying anaerobic sediment, leads to the further loss of N as N_2 gas. The growth of benthic microalgae (MPB) may be enhanced by increased light penetration to the sediment. The MPB can absorb regenerated nutrients, thereby out-competing phytoplankton. Solid lines indicate transfer of materials; dashed lines indicate diffusion of materials; dotted lines indicate microbially mediated reactions (adapted from Newell et al. 2002).

1988, Prins et al. 1995)] or as part of bivalve aquaculture, such as adjacent to rafts holding blue mussels in Spain (Tenore et al. 1982) and off-bottom aquaculture of Pacific oysters, *Crassostrea gigas*, in France (Souchu et al. 2001). The most dramatic ecosystem changes have been observed, however, in areas after the rapid population growth of an exotic species of bivalve. In San Francisco Bay, California, for example, phytoplankton has been shown to be controlled by non-native bivalves, including *Tapes japonica* and *Musculus senhousia* (Cloern 1982, Officer et al. 1982), and *Potamocorbula* spp. (Carlton et al. 1990). In freshwater systems of North America, non-native zebra mussels, *Dreissena polymorpha*, have increased to such an abundance that they have greatly reduced turbidities by consuming high levels of phytoplankton (MacIsaac et al. 1999, Strayer et al. 1999).

Seagrasses were once a very abundant ecotype in many locations worldwide, where they provided spatially heterogeneous habitat used by sessile and mobile fauna (Orth et al. 1984). These beds of submerged aquatic vegetation (SAV) have declined in many locations due to the adverse effects of anthropogenic nutrient enrichment (Twilley et al. 1985, Cloern 2001). Enhanced turbidity associated with high phytoplankton biomass can reduce the photosynthetically active radiation (PAR) to below the level required to enable SAV to grow (Twilley et al. 1985, Taylor et al. 1995).

Bivalves, by filtering phytoplankton and other particles from the water column, may increase PAR penetration to the point where SAV beds can become reestablished. The importance of suspension-feeding bivalves in promoting the reestablishment and growth of SAV has been demonstrated in the Potomac River, MD, USA. There, freshwater asiatic clams, *Corbicula fluminea*, reduced phytoplankton stocks (Cohen et al. 1984) and helped SAV to once again become dominant primary producers in freshwater locations (Phelps 1994). After a crash in the asiatic clam population of the Potomac River, there was a concomitant decline in SAV abundance (Phelps 1994). Increased water clarity will also promote the growth of microphytobenthos (MPB). These benthic algae are an important food source for both sessile and mobile benthic herbivorous meiofauna and macrofauna (Miller et al. 1996) that, in turn, are eaten by many carnivorous fish. Consequently, an abundant MPB community can support higher trophic levels.

Two modeling exercises support the idea that bivalve suspension-feeders can improve water clarity to the point that production of benthic plants is enhanced. Ulanowicz and Tuttle (1992) used an ecosystem model to predict that an important consequence of an increase in eastern oyster stocks in Chesapeake Bay would be to increase light penetration to bottom sediments to such an extent that MPB production would be greatly enhanced. Newell and Koch

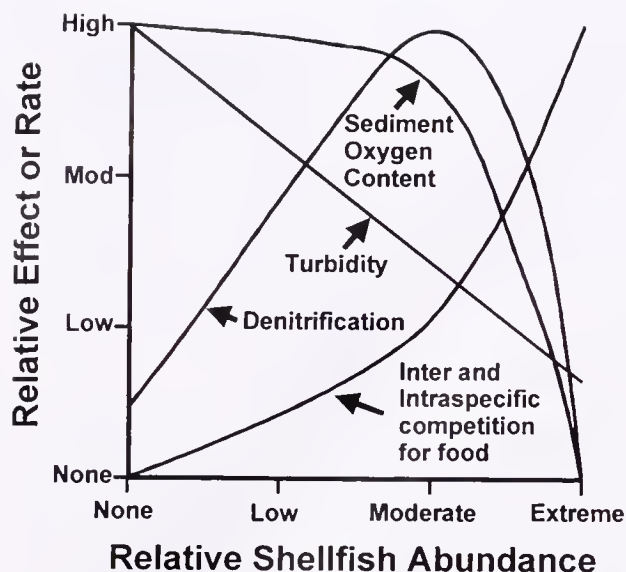


Figure 2. Conceptual figure illustrating how increasing abundances of bivalves feeding on phytoplankton and inorganic particles reduce water column turbidity in eutrophic coastal waters. At high bivalve abundances, this may lead to intra- and interspecific competition for phytoplankton with other suspension-feeding herbivores. Biodeposits, containing undigested POM, are deposited to the sediment surface where they are microbially metabolized, thereby affecting sediment oxygen content. In locations where there is either extremely heavy biodeposition or little water flow and mixing, sediment oxygen content may decline, thereby reducing coupled nitrification-denitrification.

(2004) developed a numerical model to simulate the interaction between wave-induced sediment resuspension, bivalve filtration, and seagrass growth. This model, which is parameterized based on direct measurements of eastern oyster filtration and seagrass wave dampening effects, shows that the presence of eastern oysters can reduce suspended sediment concentrations by nearly an order of magnitude. This resulted in an increase in water clarity and, hence, the depth to which seagrasses could grow. It should be noted, however, that in coastal waters and tidally flushed estuaries, the exchange of suspended particles from adjacent waters means that the localized enhancement of bivalve stocks may not reduce turbidities sufficiently to permit seagrasses to grow.

A potential adverse effect of an increase in PAR at the sediment surface is that macroalgae may become established, rather than a more normal flora of seagrasses and MPB. Some types of macroalgae (e.g., *Ulva* spp., *Enteromorpha* spp., and *Cladophora* spp.) flourish in locations that have elevated levels of inorganic nutrients and relatively low irradiances (from 18 to 175 $\mu\text{mol m}^{-2}\text{s}^{-1}$), and under such conditions can out-compete other macroalgae (Peckol & Rivers 1995, Taylor et al. 2001). In some locations, these nuisance species grow so profusely that they restrict water flow and cause sediment hypoxia when they decay (Peckol & Rivers 1995, Raffaelli et al. 1998).

INFLUENCE OF EUTROPHICATION ON BIVALVE POPULATIONS

It is conceivable that increased phytoplankton biomass associated with nutrient enrichment may be beneficial to bivalve suspension-feeders. However, it is now recognized that anthropogenic inputs of N and P alter the ratio of these inorganic nutrients from

the typical Redfield ratio of 16:1 (Malone 1992, Conley 1999, Cloern 2001). Although subject to substantial variation, N:P ratios of agricultural run-off are of the order 50:1 to 70:1, whereas sewage input is closer to 10:1. The optimal ratio of N:P for algal growth is species specific, so changes in the N:P ratio alters the competitive interaction between phytoplankton species (Rhee 1978). For example, Terry (1982) demonstrated that *Pavlova lutheri*, which is considered a nutritious species for bivalves, has a high rate of P uptake and storage, but NO_3^- uptake is inhibited by increased amounts of P in the water. Consequently, a low N:P ratio might not stimulate the growth of *P. lutheri*, hence causing it to be out-competed by less nutritious species in which NO_3^- uptake is unaffected by P concentrations.

In coastal waters subject to anthropogenic nutrient loading, dissolved silicate frequently becomes depleted in relation to N and P, thereby leading to a reduction in the proportion of total primary production contributed by diatoms (Conley et al. 1993). Diatom species are generally considered to be a highly nutritious class of microalgae, and many species have been shown to promote survival and growth of bivalves (Langdon & Newell 1996). In Chesapeake Bay after the crash of the spring diatom bloom, other less nutritionally valuable classes of phytoplankton, including cyanobacteria and dinoflagellates, become dominant possibly due to the lack of available silica (Malone 1992, Conley 1999, Cloern 2001).

In addition to the ratio of inorganic nutrients, the chemical form of a nutrient can have a profound effect on phytoplankton species composition. Ryther (1954) concluded that eastern oyster stocks in the South Shore Bays of Long Island, NY, were adversely affected in the 1950s by extensive blooms of picoplanktonic algae, including *Nannochloris atomus* and *Stichococcus* spp., that were stimulated by duck farm fecal effluent containing high levels of ammonia and uric acid. These picoplanktonic species displaced nanoplanktonic algae that are more nutritious for oysters, with the consequence that the overall food quality of the phytoplankton for eastern oysters was reduced. Currently within the same South Shore Bays there are periodic blooms of the harmful picoplankton *Aureococcus anophagefferens* that causes "brown tide" (Bricelj & Lonsdale 1997). Gobler et al. (2002) reported that this species has a competitive advantage over other phytoplankton species because it can metabolize the high ambient levels of dissolved organic nitrogen and carbon that today are derived from human rather than duck farm waste. *Aureococcus anophagefferens* is toxic to bivalves as it has been shown to reduce the feeding and growth of all life stages of many species of bivalves [reviewed by Bricelj & Lonsdale (1997)]. It is thought that such blooms of toxin-producing algae are increasing in distribution and frequency worldwide, and anthropogenic nutrient enrichment is a likely causal factor (Shumway 1990, Cloern 2001). Even if the algal toxins do not adversely affect the benthic suspension-feeders themselves, they can be bioaccumulated in commercially valuable shellfish to levels that are unsafe for human consumption (Shumway 1990).

INFLUENCE OF BIVALVES ON PHYTOPLANKTON

As summarized above, suspension-feeding bivalves can serve to improve water quality in eutrophic estuaries by exerting top-down control on phytoplankton populations. In waters with substantial rates of bivalve grazing, however, larger nanoplankton cells will be preferentially removed in comparison with smaller (<3- μm diameter) picoplankton species that are retained less efficiently on the gill of most bivalve species. Furthermore, it appears

as if the growth of picoplankton compared with nanoplankton species is favored by warmer waters and changes in the relative abundance of inorganic and organic nitrogen (Malone 1992, Gobler et al. 2002). Hence, selective bivalve feeding, which is most intense during warmer months when bivalves are feeding actively, reinforces seasonal successional cycles in phytoplankton species composition, leading to the situation where picoplankton, including cyanobacteria, become relatively more abundant than larger species in areas with shellfish populations (Prins et al. 1998). Interestingly, Souchu et al. (2001) reported that high levels of bivalve aquaculture in a poorly flushed lagoon in the Mediterranean favored the production of picoplankton in all seasons except summer. Even though bivalve grazing was most intense in summer, high levels of DIN regenerated by the bivalves were sufficient to allow even the relatively slow growing nanoplankton to grow fast enough to overcome grazer control. Based on these observations, it is apparent that even though the exact seasonal changes in phytoplankton species composition are difficult to predict, bivalve grazing may possibly adversely affect food quality for other suspension-feeders.

In nutrient-enriched systems with consequent high levels of primary production, intra- and interspecific competition for food will likely be minimal at low and intermediate levels of bivalve abundance (Fig. 2). However, in systems that are either less productive, have limited water circulation, or have very high levels of bivalve biomass, intra- and interspecific competition for food may occur between natural and aquaculture stocks of bivalves. Interspecific food competition between high abundances of bivalves and zooplankton is also possible. Lam-Hoai et al. (1997) and Lam-Hoai and Rougier (2001) reported that in areas with shellfish aquaculture, there were appreciable differences in microzooplankton (cells 40- to 300- μm diameter) community structure compared with areas with no aquaculture farms. They ascribed these differences to microzooplankton being directly grazed by bivalves and also by the suspension-feeding invertebrates attached to the aquaculture structures. Bivalves may also out-compete zooplankton for phytoplankton because bivalves over-winter as adults and are able to start feeding when water temperatures reach the threshold necessary to promote an active metabolism. In contrast, temperate copepod species, which form a dominant component of the zooplankton, rely on a relatively small number of adults to survive over-winter and which can then feed and reproduce to rebuild the population. Copepod fecundity is directly related to food availability (White & Roman 1992), and consequently, if the majority of the phytoplankton is being consumed by adult bivalves, then copepod populations will diminish. This suggests that there is likely to be an exponential increase in interspecific competition for food as bivalve stocks increase in a location from low to high levels (Fig. 2).

NUTRIENT REGENERATION

In addition to the direct "top-down" control that bivalves can exert on phytoplankton stocks, they may also exert "bottom-up" control by changing rates and processes of nutrient regeneration (Fig. 1). Bivalves, by virtue of their high clearance rates, filter phytoplankton from large volumes of water. This has the effect of focusing nutrients that are then regenerated in the sediments around the population, hence increasing nutrient concentrations within that zone. Nonetheless, the total amount of nutrients regenerated directly by bivalve excretion and the microbial degradation

of their biodeposits cannot be any greater than if the phytoplankton was being degraded solely by pelagic organisms. Consequently, maximum phytoplankton standing stock supported by the nutrients regenerated through bivalve populations cannot exceed the level that can be sustained by ambient nutrients (Newell et al. 2005). This is in distinct contrast to fin-fish aquaculture, where new nutrients in the form of fish food are continually being added to the aquaculture site. Dissolved N and P excreted by fin-fish, together with that regenerated from their fecal waste and uneaten food, can stimulate excessive phytoplankton biomass. Furthermore, residual POM from the fin-fish food and feces settling to the sediment surface can cause sediment anoxia, thereby altering benthic community composition (Gowen & Bradbury 1987, Tsutsumi 1995).

Measured rates of NH_4^+ flux from natural bivalve communities (direct excretion plus regeneration from biodeposits in the sediments) can be substantial, ranging from ~ 1 to $5 \text{ mmol N m}^{-2} \text{ h}^{-1}$ (Dame et al. 1989, Asmus & Asmus 1991, Dame et al. 1991a, Dame et al. 1992), with rates being greater in summer than in winter months (Dame et al. 1992). The nitrogen released comes not only from ingested phytoplankton but also non-phytoplankton material, such as N-rich bacteria and flagellates (Asmus & Asmus 1991), that are readily captured by bivalves (Bayne & Hawkins 1992, Kreeger & Newell 2001). These high levels of NH_4^+ regeneration have been used by some investigators as evidence that bivalve populations may not be able to exert long-lasting top-down control on phytoplankton populations. Instead, it has been suggested that bivalves serve to recycle rapidly nutrients, thereby enhancing rates of primary production and phytoplankton biomass. Such conclusions are based either on direct measurements of phytoplankton production or production potentially supported by measured rates of N flux (e.g., Dame et al. 1984, Doering et al. 1986, Doering et al. 1987, Dame & Dankers 1988, Dame et al. 1989, Prins & Smaal 1990, Asmus & Asmus 1991, Dame et al. 1991b, Dame et al. 1992, Dame & Libes 1993, Yamamuro & Koike 1993, Nakamura & Kerciku 2000, Souchu et al. 2001).

What is frequently overlooked in such studies is that the burial of N and P and removal of N from the ecosystem via denitrification is enhanced by bivalve biodeposition (Newell et al. 2002, Newell et al. 2005). When bivalve biodeposits settle to the sediment surface, any remaining PON is subject to microbial degradation that can lead to some NH_4^+ being regenerated to the water column (Fig. 1). Some N that is not microbially metabolized can become buried in the accumulating sediments (Kaspar et al. 1985, Kautsky & Evans 1987, Deslous-Paoli et al. 1992, Hatcher et al. 1994). If the surficial sediments contain sufficient oxygen, then aerobic nitrifying bacteria can oxidize nitrogen compounds within the biodeposits to NO_2^- and NO_3^- . Some of this NO_2^- and NO_3^- diffuses out of the sediment and enters the water-column dissolved inorganic nitrogen (DIN) pool and some diffuses down into the underlying anaerobic sediments. Within the anaerobic sediments, denitrifying bacteria reduce the NO_2^- and NO_3^- to N_2 gas (Henriksen & Kemp 1988, Risgaard-Petersen et al. 1994). Absent N-fixation, this gaseous N_2 is in a form unavailable to plankton and so it passes to the atmosphere without stimulating further primary production. Denitrification can only occur where there is a close juxtaposition between oxygenated conditions that support nitrifying bacteria and anaerobic conditions that support denitrifying bacteria (Kaspar et al. 1985, Kristensen 1988).

The influence of bivalve feeding and biodeposition on denitrification rates under natural field conditions have not yet been fully characterized. This is mainly due to the fact that a suitable method

for easily measuring denitrification in undisturbed sediments has only been recently devised (Cornwell et al. 1999). Newell et al. (2002) used this new method in a laboratory-based experimental system to study nitrogen regeneration from bivalve biodeposits under different scenarios typical of eutrophic estuaries. This involved using algal paste as an experimental analog of oyster biodeposits that was added to the surface of defaunated and homogenous natural sediments held in tubular cores. This was considered a realistic approach because bivalves only digest and absorb ~50% of the filtered particulate nitrogen, and hence the voided biodeposits contain a large proportion of residual PON (Newell & Jordan 1983). In these experiments, when PON was regenerated aerobically in the absence of light, 17% to 24% of the total PON added to the sediments was released as N_2 gas and hence would be unavailable to support further phytoplankton production. Newell et al. (2002) found that when the same amount of PON was degraded aerobically in incubations with sufficient light to sustain an active MPB community, little nitrogen was released to the water column and there was net N_2 fixation. It is widely recognized that an actively growing MPB community can limit nitrogen fluxes across the sediment-water interface (e.g., Sundbäck & Graneli 1988, Krom 1991, Rysgaard et al. 1995, Cerco & Seitzinger 1997, Sundbäck et al. 2000). Nitrogen fixation within MPB communities has previously been reported in many shallow-water environments when N requirements of benthic algal photosynthesis exceeds that supplied from the water column and regenerated from the sediment (Joye & Paerl 1994).

In addition to the role of MPB in intercepting DIN being regenerated from the sediments, the oxygen produced from MPB photosynthesis can alter sediment biogeochemistry. Some of this oxygen diffuses down into the sediment, thereby inhibiting the buildup of reduced inorganic compounds (Epping et al. 1999). In sediments subject to bivalve biodeposition, oxygen released by MPB can be used by bacteria in the micro-zone at the sediment-water interface to maintain nitrification, which is the critical precursor to denitrification (Sundbäck et al. 1991, Risgaard-Petersen et al. 1994, Rysgaard et al. 1994, An & Joye 2001). This pattern changes when MPB growth is high, such as may occur when turbidities are reduced by bivalve feeding in shallow-water locations. Under such circumstances, the demand for inorganic nutrients by MPB can be so great that they out-compete the nitrifying bacteria in the aerobic sediments for NH_4^+ . This results in a decline in the production of NO_2^- and NO_3^- , thereby curtailing coupled nitrification-denitrification (Risgaard-Petersen et al. 1994, Rysgaard et al. 1995, An & Joye 2001, Newell et al. 2002).

In one of the few field studies of the role of bivalves in nutrient cycling in which denitrification was evaluated, Kaspar et al. (1985) found appreciably higher denitrification in sediments underlying rope-cultured mussels than at reference sites. [Note that denitrification was measured in this study using the acetylene block technique that provides information on denitrification potential rather than absolute rates (Cornwell et al. 1999)]. Kaspar et al. (1985) discovered that biodeposition from aquacultured mussels not only increased PON in the sediment but also changed the benthic community from a diverse assemblage, including epibenthic species, to one composed solely of infaunal polychaetes that enhanced bioturbation and hence increased coupled nitrification-denitrification. Hatcher et al. (1994) reported enhanced sedimentation under mussel aquaculture ropes that caused an increase in N accumulation in the sediment, a shallowing of the Redox Potential Discontinuity layer, and enhanced the NH_4^+ efflux from the sediment compared

with adjacent control areas. Such changes in sediment biogeochemistry do not preclude the possibility that there was enhanced denitrification in the sediments. Rather, as Hatcher et al. (1994) state, they could not make any definitive statements concerning the influence of bivalve biodeposition on ecosystem nitrogen balance because they did not have the techniques available to measure denitrification.

Souchu et al. (2001) studied how bivalve aquaculture altered water column nutrient cycling in a poorly flushed lagoon in the Mediterranean. They reported that for all seasons, except when phytoplankton were growing most rapidly in summer, bivalve grazing controlled phytoplankton biomass. Consequently, for most of the year the regenerated NH_4^+ from the aquaculture farms was not used by phytoplankton for new production. Instead it became available to be oxidized to NO_3^- by pelagic nitrifying bacteria, hence explaining their observation of elevated NO_3^- in the water column within the shellfish aquaculture farms. It is likely that this NO_3^- will diffuse into the sediments where it will be subject to denitrification, hence leading to N removal from these coastal lagoons.

Although bivalves are clearly important mediators of N cycling, their role in P cycling is less clear. For example, some studies demonstrate that P regeneration from bivalve stocks does not increase (Dame et al. 1989, Dame et al. 1991) or increases by ~50% (Asmus & Asmus 1991, Souchu et al. 2001). The balance between binding and release of P from oyster biodeposits is highly dependent on sediment oxygenation and the development of a redox gradient within the sediments. In estuarine sediments, P fluxes are controlled by interfacial adsorption and desorption processes, often involving iron and sulfur cycling (Krom & Berner 1981). Iron oxides at the sediment-water interface are a diffusive barrier to P fluxes across the sediment-water interface. Under fully oxygenated conditions, any P remaining in the biodeposits will be buried in the accumulating sediments. If the depth of the oxygenated zone in the surficial sediments decreases, the P-adsorbing iron oxide surface area can be reduced to iron monosulfides, thereby allowing the release of sedimentary inorganic P (Boynton & Kemp 1985).

The ecosystem effects of an increase in bivalves on sediment nutrient regeneration, and hence on phytoplankton production, will vary depending on bivalve population density and the rate of mixing of oxygenated water down to the sediment surface. Excess biodeposition, especially in low water flow environments, has the potential to stimulate bacterial respiration to such an extent that sediments become anoxic, thereby inhibiting coupled nitrification-denitrification (Fig. 2) and causing sediment-bound P to be mobilized. Such local adverse effects can be ameliorated by moderate water currents or wave action that allows biodeposits to be spread across a larger bottom area and that mix oxygen from the surface to the bottom waters (Haven & Morales-Alamo 1968, Dame et al. 1991).

The adverse effects of sediment overenrichment by bivalve biodeposits have often been observed in sediments underlying bivalves in suspended raft culture. For example, Ito and Imai (1955) reported that intensive oyster aquaculture resulted in underlying sediments becoming anoxic, and these effects appeared cumulative because the longer oysters were cultivated in a location, the more frequently sediment anoxia occurred. Such reductions in sediment oxygen content will reduce rates of bacterially mediated nitrification and increase the proportion of N released as NH_4^+ . When sediments become completely anoxic, the buildup of H_2S can kill

the aerobic nitrifying bacterial community. Consequently, even if aerobic conditions in the surface sediments are restored, nitrification will only recommence following the regeneration of the nitrifying bacterial community (Henriksen & Kemp 1988, Sloth et al. 1995). Similarly, sediments underlying intensive mussel raft aquaculture in a Spanish Ria exhibited rates of sulfate reduction, indicative of anaerobic microbial processes, that were 63% greater than in sediments outside the raft area (Tenore et al. 1982). Tuttle and Jonas (1992) also observed elevated amounts of microbially labile organic matter in surficial sediments beneath eastern oysters grown in floats in Chesapeake Bay. This led to about a 4-fold increase in sulfate reduction rates, although this increase was short-lived and confined to sediments in the immediate vicinity of the floats. These findings suggest that extremely dense bivalve communities can adversely affect sediment microbial processes by shifting them from aerobic to anaerobic metabolism as a result of increased POM loading (Fig. 2).

The development of anoxic sediments associated with intensive bivalve aquaculture can increase hydrogen sulfide to levels that are toxic to benthic animals (Diaz & Rosenberg 1995). For example, Dinert et al. (1990) studied bivalve aquaculture sites and observed that as biodeposition by *Crassostrea gigas* and *Mytilus edulis* increased, there was a commensurate decline in meiofaunal populations associated with sediment anoxia and elevated NH_4^+ in sediment pore water. Declines have also been observed (Tenore et al. 1982, Rodhouse & Roden 1987) in the abundance and species diversity of the burrowing and deposit-feeding macrobenthic organisms (bioturbators) that actively mix surficial sediments as a result of their feeding and burrow irrigation activity. Bioturbation serves to increase the surface area to volume ratio of the oxic-anoxic sediment interface both by forming anoxic microenvironments within the upper aerobic zone and by driving the redox potential discontinuity deeper into the sediments (Kristensen 1988). In this spatially complex interface between aerobic and anaerobic zones, denitrification rates are enhanced compared with sediments without bioturbators (Kaspar et al. 1985, Henriksen & Kemp 1988, Kristensen 1988, Pelegri et al. 1994).

Dense assemblages of bivalves do not always cause adverse changes in benthic community structure. For example, Dittmann (1990) reported that biodeposition from beds of blue mussels leads to an enhanced and more diverse benthic invertebrate assemblage that will promote bioturbation. In addition, POM remaining in biodeposits provides a major source of food to benthic meio- and macrofauna and serves as a food resource for secondary consumers. Grant et al. (1995) found relatively minor changes in macrobenthic biomass and diversity associated with biodeposition from suspended mussel culture. In contrast, Kaspar et al. (1985) found that the benthic community underlying rope-cultured mussels changed from one with a diverse species composition to one composed solely of infaunal polychaetes. As noted earlier, however, this increase in bioturbating polychaetes was responsible for enhancing the coupled nitrification-denitrification found in the aquaculture sediments compared with the control sediments.

THE POSSIBLE USE OF BIVALVES IN EXTRACTIVE AQUACULTURE

Current management strategies for curtailing the adverse effects of eutrophication in coastal waters depend on upgrading sewage treatment facilities, reducing agricultural run-off, and control-

ling atmospheric inputs (D'Elia et al. 1992). It is now recognized that a possible supplement to such nutrient controls is "extractive aquaculture" in which macroalgae are grown both for their biomass and the concomitant removal of the nutrients from that impaired water body (Chopin et al. 2001). The harvested biomass can be used either for food (e.g., the food additive carrageenan and *Porphyra* spp. used to make "nori") or as agricultural compost for fertilizer and biogas production (Gao & McKinley 1994).

Another heretofore not widely recognized form of extractive aquaculture is the growth of suspension-feeding bivalves. On a dry-weight basis, eastern oyster tissue and shell contains nitrogen (~7% and ~0.3%, respectively) and phosphorus (~0.8% and ~0.1%, respectively) (Galtsoff 1964; Newell, unpublished data) that is removed from the ecosystem at harvest. For example, a market-size eastern oyster of shell length 7.6 cm has a shell that weighs ~150 g and ~1 g dry tissue and which combined removes a total of 0.52 g N and 0.16 g P at harvest. Much of this N and P is in the relatively large shell and so when species with lighter shells, such as blue mussels, are harvested, less N and P will be removed.

In addition to the direct removal of N and P by harvesting, microbially mediated denitrification of PON transferred to the sediment surface in bivalve biodeposits has the beneficial effect of permanently removing N in the form of N_2 gas from the ecosystem. Burial of residual N and P from bivalve biodeposits in sediments also enhances nutrient removal from the water column. Consequently, suspension-feeding bivalves potentially offer this additional mechanism for N and P removal compared with extractive aquaculture of algae. Newell et al. (2005) estimated that the biodeposition activity of an adult eastern oyster (shell height 7.6 cm; ~1 g dry tissue weight) feeding under natural conditions in the Choptank River, a mesohaline tributary to Chesapeake Bay, caused 0.27 g P to be buried and 0.75 g N to be buried and denitrified annually. These estimates did not take into account the reduced levels of N and P removal during the time it takes oysters to grow from juvenile to adult size nor the enhanced levels of N and P removal as oysters grow larger than 7.6 cm.

One of the reasons for the increasing interest in extractive aquaculture is the possibility of a nutrient trading system being implemented in coastal waters, such as Chesapeake Bay (www.chesapeakebay.net/trading.htm). Nutrient trading schemes allow industry and municipal waste-water treatment facilities to purchase at market value extra nutrient removal capacity from others on the same receiving water body, rather than directly reducing their own nutrient inputs. This is becoming an important issue in the United States, where the Environmental Protection Agency is mandated by the Clean Water Act to improve water quality. One mechanism devised to help reduce phytoplankton production and thereby enhance water quality is the total maximum daily load (TMDL), which is the maximum daily amount of nutrients allowed to be discharged into a water body from all sources (www.epa.gov/waterscience/standards/nutsi.html). Nutrient trading will allow aquaculturists, who can document the amount of N and P removed by their farms, to be paid by those industries that find it less expensive to purchase nutrient removal rather than upgrade their own facilities to meet the TMDL. The use of extractive animal and plant aquaculture is actually a unique solution to helping attain these water-quality standards because it offers the only opportunity to reduce nutrients once they have entered a receiving body of water. This may be especially important in ameliorating the effects

of non-point source inputs that are the most difficult to regulate and control.

Aquaculture farms will have to undertake some procedures beyond those required for successful shellfish production to obtain remuneration for the amounts of N and P removed beyond those incorporated in bivalve tissue and shell. Such changes are necessary because sediment nutrient regeneration is extremely dependent on how the aquaculture facility is operated, including the species of bivalve being cultivated and the stocking densities. Furthermore, seasonal and physical characteristics of the aquaculture site, such as sediment porosity, water flow, abundance of bioturbators, and so forth, can directly and indirectly influence sediment biogeochemical processes. Consequently, any financial gain associated with nutrient trading will have to be balanced against possible increased operating costs associated with obtaining the targeted rates of nutrient removal.

The maximum top-down control on phytoplankton and enhancement of N and P removal only comes from species of bivalves, such as oysters, that maintain high clearance rates even when seston concentrations increase. Other species, such as hard clams, because of their comparatively low clearance rates (Grizzle et al. 2001, Newell & Koch 2004), have less influence on benthic pelagic coupling. In eutrophic conditions, the most active suspension-feeders will reject large amounts of phytoplankton as part of their copious pseudofeces that are then transferred to the sediment surface. These receiving sediments must always remain fully oxygenated because microbially mediated nitrification only occurs under aerobic conditions, and this is the crucial precursor to denitrification in the underlying anaerobic sediments. Similarly, in fully oxygenated sediments, but not anaerobic sediments, phosphorus can become bound to iron and remain buried in the accumulating sediments. Therefore, bivalve stocking densities must be constrained to prevent local overenrichment of the sediments by the biodeposits that can lead to such intense microbial activity that even the surface sediment becomes anoxic (Fig. 2). Ideally, bivalves should be cultivated in locations with moderate current flow because the continual supply of oxygenated water reduces the likelihood of bottom sediments becoming anaerobic. Water currents also cause the biodeposits to be distributed across a larger bottom area (Haven & Morales-Alamo 1968), further reducing the likelihood that the surface receiving sediments will become anoxic. Water flow also increases seston flux through the aquaculture site, thereby ensuring an adequate food supply to the bivalves (Wildish & Kristmanson 1997).

Because some of the ecological benefits associated with aquaculture of bivalve suspension-feeders are so dependent on having aerobic sediments, the degree of sediment oxygenation around the aquaculture site must be regularly monitored. This can be performed routinely by measuring the depth of the redox potential discontinuity (RPD), either in sediment cores collected in transparent acrylic plastic tubes (Grizzle & Penniman 1991) or by using a remotely operated camera system that directly photographs the sediment profile (O'Connor et al. 1989). If the depth of the RPD is found to be moving progressively shallower, it indicates that the dynamic balance between aerobic and anaerobic sediments is shifting toward less oxygenated sediment. In such a situation, remedial actions should be taken to maintain the aerobic conditions needed to sustain both nitrifying bacteria and the benthic bioturbator community. This might require the use of a paddle-wheel system to increase the rate of mixing of surface oxygenated water to the bottom. Alternatively, if off-bottom aquaculture systems are being

used, these could possibly be relocated until the normal depth of the RPD at the original site is restored.

The reduction in turbidity associated with bivalve feeding may be sufficient to increase PAR penetration to the sediment surface to a level that can sustain MPB production. Actively growing MPB absorb inorganic nutrients released from bivalve biodeposits at the sediment water interface and compete with the sediment microbial community for N remaining in the biodeposits. Consequently, in locations where sufficient PAR reaches the sediment surface to permit active MPB growth, bivalve biodeposition may not result in appreciable nitrogen removal via denitrification (Newell et al. 2002). Therefore, if enhanced denitrification is a desired ecosystem service, aquaculture stocks of bivalves should be cultivated in deeper locations where the biodeposits will be transferred to sediments beneath the euphotic zone.

In any nutrient trading scheme involving suspension-feeding bivalves, it will be relatively easy to account for N and P removed in shell and flesh based on the annual harvest levels. Unfortunately, the factors that govern the magnitude of N removal and P immobilization (Newell et al. 2005) that are by-products of rearing bivalves are too complex and variable to allow the use of fixed removal rates that can be applied across all shellfish aquaculture facilities. For example, seasonal and physical characteristics of the aquaculture site, such as sediment porosity, current flow, abundance of bioturbators, and so forth, can directly and indirectly influence sediment biogeochemical processes. The magnitude of these possible variations are illustrated in a study by Sundbäck et al. (2000) of two different grain size sediments. They reported that coupled nitrification-denitrification rates were about an order of magnitude higher annually in finer grain sediments with active bioturbators than in sediments of higher porosity and with a slightly lower biomass of bioturbators. Because of such complex interactions, aquaculturists participating in nutrient trading will have to document the actual N and P removal at the specific locations. Appropriate N and P flux measurements can be made in sediment cores collected seasonally from the aquaculture sites and adjacent control sites. For shallow-water sites, these incubations will have to be made both in darkness and in the light to account for the influence of MPB on sediment biogeochemistry. Membrane inlet mass spectrometry can be used to obtain precise measurements of denitrification rates in relation to the release into the water column of other forms of inorganic nitrogen (Cornwell et al. 1999, Newell et al. 2002). The accumulation and burial of N and P into the sediment can be measured in separate sediment cores. These measured N and P removal rates can then be used as the basis of deciding what level of remuneration may be obtained from nutrient trading.

ACKNOWLEDGMENTS

I am grateful to Dr. Jeff Cornwell and Dr. Tom Fisher for their scientific discussions. I would like to thank Ray Grizzle, Becky Holyoke, Carter Newell, and Bob Rheault for their reviews of an early draft of this manuscript. This research was supported with funding from Maryland Sea Grant (SA07528051-F) through NOAA award NA16RG2207. The U.S. government is authorized to publish reprints of this work, and the author reserves the right to post a copy on his academic Web site for the private and noncommercial use of individuals, notwithstanding any copyright notations hereon.

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LONG-TERM PROJECTIONS OF EASTERN OYSTER POPULATIONS UNDER VARIOUS MANAGEMENT SCENARIOS

STEPHEN J. JORDAN¹ AND JESSICA M. COAKLEY²

*U.S. Environmental Protection Agency, Gulf Ecology Division, Gulf Breeze, Florida 32526; and
University of Maryland, Center for Environmental Science, Sarbanes Cooperative Oxford Laboratory,
Oxford, Maryland 21654*

ABSTRACT Time series of fishery-dependent and fishery-independent data were used to parameterize a model of oyster population dynamics for Maryland's Chesapeake Bay. Model parameters are (1) fishing mortality, estimated from differences between predicted and reported landings scaled to a fishery-independent estimate of exploitation rate; (2) natural mortality, estimated from the ratio of dead to live oysters in fishery-independent surveys; (3) recruitment, estimated from annual changes in the market oyster stock and total mortality rates; and (4) carrying capacity, estimated from predicted densities of oysters in the absence of either exploitation or excess mortality from parasitic diseases, multiplied by charted areas of high-quality and marginal habitat. The model predicts continuing decline in population abundance and biomass in the absence of significant management intervention. Moderate decreases in fishing mortality, alone or in combination with increases in recruitment through stock enhancement, could reverse recent population trends, resulting in a larger population and improved harvests within 1–2 decades, even if currently high rates of natural mortality are sustained.

KEY WORDS: *Crassostrea virginica*, fishing mortality, models, natural mortality, oyster, population dynamics, recruitment

INTRODUCTION

In 2000, the inter-jurisdictional Chesapeake Bay Program (CBP) established a goal to increase the bay's population of native oysters (*Crassostrea virginica*) 10-fold by 2010 from a 1994 baseline. With colleagues, we developed a preliminary quantification of the oyster population in Maryland's portion of the bay, along with methods for tracking population trends and predicting annual landings with an index of relative biomass (Jordan et al. 2002). A logical next step was to examine the potential contributions of various management strategies toward meeting the 10-fold restoration goal. To this end, we used time series of fishery-dependent and fishery-independent data to parameterize a model of oyster population dynamics. The model projects long-term trends in the Maryland stock of market-sized oysters (≥ 76 mm in shell height) with variable rates of fishing mortality (F), natural mortality (M), and recruitment (R). The model is limited to market oysters, first for simplicity, second because the relationship between oysters < 76 mm in shell height (pre-recruits) and the market stock has been difficult to quantify (Jordan et al. 2002), and third, because the goals of the CBP and the state of Maryland include restoration of the oyster fishery, in addition to the population as a whole (Maryland Oyster Roundtable 1993, CBP 2002). Moreover, the standing stock of larger oysters is an important indicator of population viability, whether the objectives of the restoration program are ecological, economic, or both.

The model is stochastic, that is, for each of many iterations of the model within a simulation, F, M and R vary randomly, constrained by means and standard deviations observed from the monitoring time series or specified by the user. The principal outputs are projected annual means of market oyster stocks and landings. To estimate the uncertainty associated with the results, the model computes percentages of model iterations that achieve specified targets. Thus, a user could evaluate proposed management strategies on the basis of, for example, 90% probability of achieving a desired outcome. Although the CBP oyster restoration goal set 2010 as the year in which to achieve a 10-fold increase, we

extended simulations to 2020 to present longer-range views of population trends.

Environmental forcings (e.g., variations in temperature and salinity) are not included explicitly in the model, except in the separate baseline parameter values for three salinity zones. We assume that the effects of environmental variations have been captured in the standard deviations of the baseline model parameters. The time-series data spanned 16 y, from 1986 to 2001, capturing a range of warm, cool, wet, and dry periods, along with other variations in the environment.

The model was designed to evaluate long-term management strategies in response to the CBP 10-y goal, and does not accurately reproduce or project short-term (interannual) population trends. Year-to-year tactical management decisions would be better addressed by a model similar to that developed by Klinck et al. (2001) for Delaware Bay oysters. The CBP (2002) emphasis on sanctuaries (i.e., areas permanently closed to oyster harvest) is an example of a long-term strategy appropriate for the model described here. In Maryland, sanctuaries generally are stocked with seed oysters produced in hatcheries. Consistent with the CBP plan and recent practice, we modeled the sanctuary strategy as a 10% decrease in F (to approximately 10% of productive bottom closed to the fishery), combined with a 10% increase in R (approximating potential hatchery contributions), and projected this scenario over 20 y. Another example of a long-term management strategy is projecting the effects of reductions in F in the range of 10–40%, for which the model predicts that initial decreases in landings will be more than compensated by increases after a few years.

The CBP (2002) plan is concerned with selecting sites for restoration projects and how different areas of the bay should be managed to achieve the restoration goal. The models for three salinity zones should be helpful in these decisions, although an ideal model for these purposes would have much finer geographical resolution (e.g., individual oyster bars). The model reported here should be a step toward a more detailed, age-structured, geographically articulated model.

MATERIALS AND METHODS

Parameters of the model are instantaneous rates of natural mortality (M), fishing mortality (F), and recruitment (R), plus point estimates of carrying capacity (K , an upper bound on the stock of market oysters). The unit of measure is one Maryland bushel (~ 46 L) of oysters. The only boundary condition is an initial estimate of the market stock (in bushels), which can be derived from landings and relative biomass (as described below) for any year for which monitoring data are available (Jordan et al. 2002).

The primary sources of data for model parameterization were (1) Maryland fall dredge surveys of oyster bars (1985–2000), and (2) annual commercial oyster landings reported to the Maryland Department of Natural Resources. The fall dredge surveys provided annual estimates of natural mortality rates and relative biomass of market-sized oysters and are described elsewhere (Smith and Jordan 1993, Homer et al. 1996, Maryland DNR 2001, Jordan et al. 2002).

We developed an aggregated model for all of Maryland's Chesapeake Bay and also developed separate baseline parameter values for each of three zones based on salinity data from the fall oyster surveys (Fig. 1). Salinity zones were defined by averaging salinity measurements taken from 43 dredge survey sites in October or November 1990–2000, then assigning sites to categories: high, >14 ppt; medium, 12–14 ppt; and low, <12 ppt. We used two criteria to establish the salinity ranges: (1) including roughly equal numbers of sites in each category so that each would have sufficient data, and (2) gradients in recruitment and the impacts of the oyster parasites *Haplosporidium nelsoni* and *Perkinsus marinus* on oyster mortality. At salinity <12 ppt, *H. nelsoni* infections occur rarely, if ever; *P. marinus* infections, although chronic in this zone, are associated with low to moderate mortality rates; and recruitment rates of both small and market oysters are typically very low, except in areas where natural recruitment has been supplemented by transplanted seed oysters. In the medium-salinity zone, *H. nelsoni* epizootics are sporadic, occurring only in drought years, mortality associated with *P. marinus* is moderate to high, and recruitment is variable. In the high-salinity zone, *H. nelsoni* infections tend to be enzootic, mortality rates associated with *P. marinus* are consistently high, and recruitment, although variable, tends to be higher than in the lower salinity zones (Jordan 1995, Giesecker 2001).

The model assumes classic logistic population growth, with two enhancements. First, instead of a net rate of population change [r , as in Krebs (1994)], the model includes separate parameters for instantaneous rates of natural mortality (M), fishing mortality (F), and gross annual recruitment (R). Second, each parameter is input for each simulation in Monte Carlo fashion, as a random number from a log-normal distribution with mean and standard deviation computed from the time series of monitoring data.

Natural mortality (M) estimates were based on the ratio of articulated dead oyster shells (D = boxes) to the total of live and dead oysters ($L + D$) in each dredge sample: $M = -\log_e(D/(L + D))$. Fishing mortality was estimated from the ratio of reported landings to landings predicted by log-log linear regression from a fishery-independent biomass index for market oysters: $F = -\log_e[0.53 (H/\hat{H})]$, where H = reported landings, \hat{H} = landings predicted from relative biomass of market oysters, and 0.53 is a scaling constant derived from a fishery-independent estimate of the proportion of the market stock landed during the 1990–1991 season (Smith and Jordan 1993). For that season, the ratio H/\hat{H} was

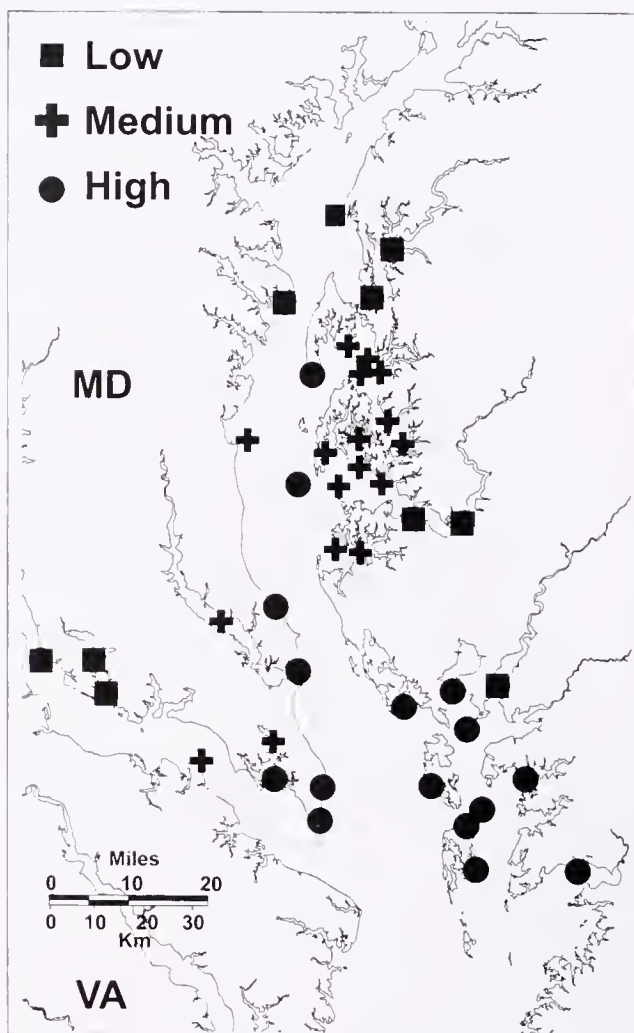


Figure 1. Northern Chesapeake Bay, showing 43 monitoring sites where oyster mortality, size frequency, and relative abundance data were collected.

approximately unity. These methods of estimating M and F were described and discussed by Jordan et al. (2002). Gross recruitment (R) was calculated as the instantaneous annual rate of change in the market oyster stock ($r = dS/dt$), plus total annual mortality ($Z = M + F$). The term dS/dt was estimated for a 1987–2001 time series by calculating stock size for each year as $S = (H)/(1 - e^{-r})$, where H is annual landings (R could not be computed for 1986, the first year of the monitoring time series).

We made four estimates of carrying capacity, one for each of the salinity zones, and one for the Maryland Chesapeake Bay as a whole. The Maryland Bay Bottom Survey (MBBS) of oyster bars was used to calculate acreage of cultch, mud with cultch, and sand with cultch bottom-type categories for each area. A variety of observations and surveys with patent tong grabs, divers, video, and acoustics indicated that only about 10% of these areas of nominal oyster habitat actually supported oyster populations (Smith et al. 2001, Maryland Department of Natural Resources, unpublished data). Even within productive areas, high densities of oysters (≥ 100 per m^2) occurred only in scattered patches. Thus, we assumed that at carrying capacity, 10% of cultch areas from the MBBS would support mean densities of 10 market oysters per m^2 .

and 10% of sand with cultch and mud with cultch areas would support mean densities of 3 market oysters per m².

We attempted to decompose Maryland-wide landings data to match the three salinity zones, but values of *F* estimated from landings and relative biomass by zone were extremely variable and in some cases unreasonable. Although landings were reported for discrete areas (fishing regions defined by the National Marine Fisheries Service) that could be matched with monitoring sites, landings reports reflected where the oysters were landed, not necessarily where they were caught. Moreover, some landings were reported from areas that could not be assigned to salinity zones based on monitoring data. Therefore, we used the bay-wide mean *F*, along with zone-specific estimates of *M*, *R*, and *K*, to initiate the salinity zone parameterizations.

The equation for simulating the market oyster population is: $S_t = (K/I + G \cdot e^{-[R-(M+F)]})$, where S_t is the market oyster stock in year *t*, $G = (K - S_{t-1})/(S_{t-1})$, S_{t-1} is the stock in the previous year, *e* is the base of natural logarithms, and the other parameters are as above. Values of any of the parameters can be specified to simulate particular scenarios. Standard deviations of *F*, *M*, and *R* also can be varied if desired. In all simulations reported here, the model was run for 20 y with 1000 iterations per year. Preliminary simulations with 5000 iterations produced results almost identical to those with 1000 iterations. Model output is processed to generate log-mean simulated stock size and landings for each year of the simulation. Simulated means of stock and landings are back-transformed to arithmetic values and graphed as time-series plots to portray expected trends. We established three reference points for evaluating bay-wide scenarios: (1) stock collapse, defined as market stock $< 0.1 \times 10^6$ bushels, (2) stock restoration, defined as a stock of at least 1.77×10^6 bushels (i.e., 10 times the stock estimate for the oyster restoration goal baseline year 1994), and (3) fishery restoration, defined as annual landings $\geq 2 \times 10^6$ bushels, consistent with the magnitude of landings sustained from the 1920s through the 1970s. The model calculates percentages of simulations within each scenario that indicate stock collapse or that achieve or exceed the fishery or stock restoration targets in the final year of simulation (2020), as measures of uncertainty for the bay-wide model. Lack of resolution in the landings data precluded fisheries targets for the salinity zone models, and starting stocks were so low in the medium- and high-salinity zones that stock restoration and stock collapse reference points would have had little meaning. We set a stock restoration reference for the low-salinity zone based on the CBP (2000) oyster restoration goal (i.e., 10 times the 1994 stock).

In the simulations reported here, we varied *F* and *R* to forecast the trends that would be expected under reasonable scenarios of management intervention (reductions in *F* and stock enhancement with hatchery-produced seed oysters). To simulate increased recruitment by means of stock enhancement, we made the following assumptions: hatchery production would not exceed about 2 to 3 $\times 10^8$ spat on shell per year; 30% survival of hatchery oysters from planting to market size; recruitment to the market stock 4 y after planting (Jordan et al. 2002); and 375 market oysters per bushel. These assumptions limited reasonable scenarios to a 10% increase in *R*. We also ran the models with baseline parameter values in both forecasting and hindcasting modes.

RESULTS

Maryland oyster landings varied by a factor of almost 20 from 1986 to 2001 (Table 1). Variations in *F* corresponded to annual

TABLE 1.
Baseline time-series data for the Maryland bay-wide model.

Year	Landings (1000 bushels)	Stock (1000 bushels)	F	M	R
1986	1560	3211	0.665	0.106	N.A.
1987	980	1428	1.159	0.501	0.850
1988	360	909	0.504	0.589	0.641
1989	400	551	1.294	0.375	1.169
1990	410	768	0.763	0.170	1.265
1991	420	816	0.723	0.217	1.001
1992	320	707	0.603	0.470	0.929
1993	120	238	0.701	0.908	0.521
1994	80	177	0.598	0.786	1.087
1995	166	321	0.730	0.276	1.602
1996	201	452	0.590	0.350	1.281
1997	177	862	0.231	0.335	1.212
1998	285	670	0.554	0.217	0.519
1999	422	678	0.976	0.202	1.189
2000	380	802	0.642	0.367	1.177
2001	348	702	0.684	0.444	0.995
Mean	414	830	0.714	0.394	1.029
SD	368	701	0.253	0.221	0.300

Year is the year in which October-March oyster landings were reported. *F*, *M*, and *R* are instantaneous rates of fishing mortality, natural mortality, and gross recruitment to the market stock, respectively. Landings data were rounded to the nearest 1000 bushels. *R* was not computable for 1986; *K* = 16,964,000 bushels of market-size oysters.

exploitation rates of 21–73% of the market stock. Natural mortality ranged from 10–60%, total mortality 43–81%, and gross recruitment 40–72%. Annual net rates of change in the market stock varied from –66% to +48%, with a majority (8 of 15 y) of negative values. The mean annual rate of change in the market stock from 1987 to 2001 was –10%. Annual landings and natural mortality (*M*) for the bay-wide model, along with computed values of *K*, *F*, *R*, and *S*, are listed in Table 1.

Because the monitoring data we used to establish salinity zones did not support full coverage of oyster habitat or reported landings, our estimates of bay-wide *K* and initial stock were greater than the sums of *K* and initial stocks for the three salinity zones. Mean baseline parameters, initial stocks, and *K* for the three salinity zones are listed in Table 2.

Means of 1000 runs of a bay-wide hindcast simulation using the baseline parameters reproduced the observed trends in stock and landings from 1986 to 2001, but not year-to-year variation

TABLE 2.
Model baseline parameters and initial stock estimates for three salinity zones.

Zone	Low	Medium	High
<i>K</i>	4221.8	3006.4	5034.8
Initial stock	231.9	488.9	39.4
<i>F</i>	0.714 (0.253)	0.714 (0.253)	0.714 (0.253)
<i>M</i>	0.165 (0.081)	0.428 (0.287)	0.719 (0.519)
<i>R</i>	0.739 (0.589)	1.1885 (1.046)	1.283 (2.105)

The units of *K* and initial stocks are bushels $\times 10^3$. Means of *F* (simulated) and *M* are from 1986 to 2000 time series; the mean of *R* is from 1987 to 2000. Standard deviations are in parentheses.

(Fig. 2). Many individual iterations from this simulation generated patterns similar to the actual time series, but many others diverged widely. The two examples shown in Figure 3 illustrate the stochastic properties of the model. The single iteration of the model shown in the upper pane of Figure 3 generally matched the pattern and magnitude of reported landings, whereas the iteration shown in the lower pane of Figure 3 was an extreme deviation from the data.

Forward simulation with baseline parameter values in the bay-wide model indicated continuing declines in stock and landings through 2020. A reduction of fishing mortality to 0.9F (90% of baseline, or $F = 0.64$) moderated, but did not reverse these trends. Forward simulations produced a consistent pattern of long-term increases in stock and landings at 0.8F and lower (Fig. 4). Because of overlapping trends among the scenarios, projected landings can be seen more clearly in three dimensions (lower plot of Fig. 4). At 0.8F ($F = 0.57$), the model projected approximately linear increases in stock and landings, with modest reductions in landings below the 1.0F and 0.9F scenarios for the first few years. Initial reductions in landings were followed by increases in subsequent years. At 0.7F ($F = 0.50$), there were exponential increases in stock and landings, with the stock increasing by a factor of 5.5 and landings by 5-fold over 20 y. Early losses to the fishery were compensated within the first few years. Reducing F to 0.6F ($F = 0.43$) increased stock by more than 10-fold and landings by 9-fold. Greater reductions in F (50% or more) increased stock size more rapidly, but long-term landings were less than for the 0.6F scenario. Simulated long-term fishery yields were optimum at 0.6F ($F = 0.43$; Fig. 4).

Forty-three percent of bay-wide simulations with 1.0F predicted stock collapse to $<0.1 \times 10^6$ bushels (Fig. 5). Reducing F to

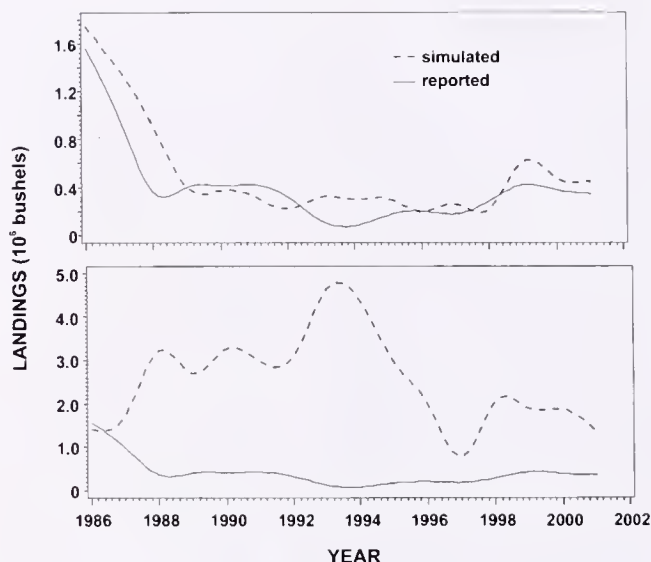


Figure 3. Simulated landings from two individual iterations of a hindcast of the bay-wide oyster model with baseline parameter values compared with reported landings; smoothed by polynomial spline interpolation.

0.6F or less virtually eliminated the probability of stock collapse. Stock restoration ($\geq 1.77 \times 10^6$ bushels) was achieved in 9.2% of simulations at 1.0F and 98% of simulations at 0.5F. The most stringent target, fishery restoration ($\geq 2 \times 10^6$ bushels projected landings), was achieved in 73% of simulations for 0.4F and 0.5F (right panel of Fig. 5); greater or smaller reductions in F indicated less likelihood of achieving the fishery restoration target.

Increasing the instantaneous rate of recruitment by 10% (1.1R, the maximum we thought to be achievable based on realistic levels of hatchery production), with F held at 1.0F, stabilized stock and

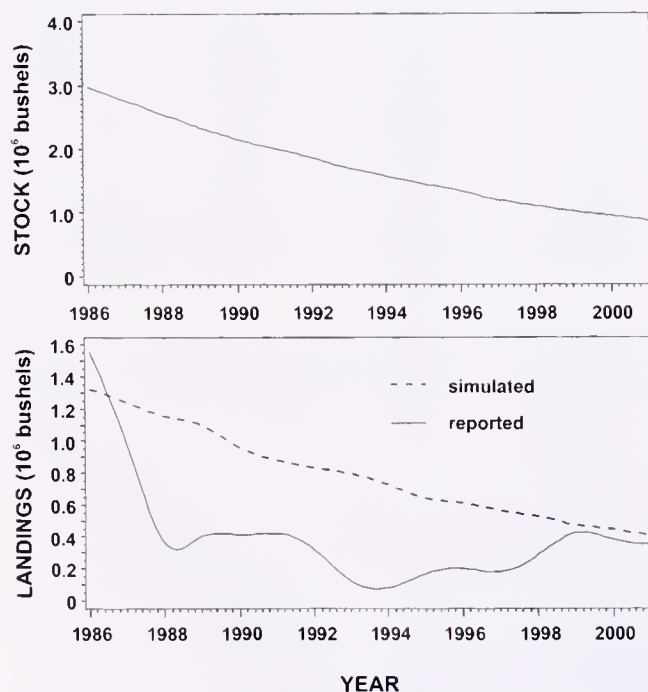


Figure 2. Mean market oyster stock and landings simulated from a hindcast of the bay-wide oyster model with baseline parameter values. Reported landings are shown on the lower plot; no model-independent estimates of stock size were available for comparison. Simulated annual means and reported annual landings were smoothed by polynomial spline interpolation.

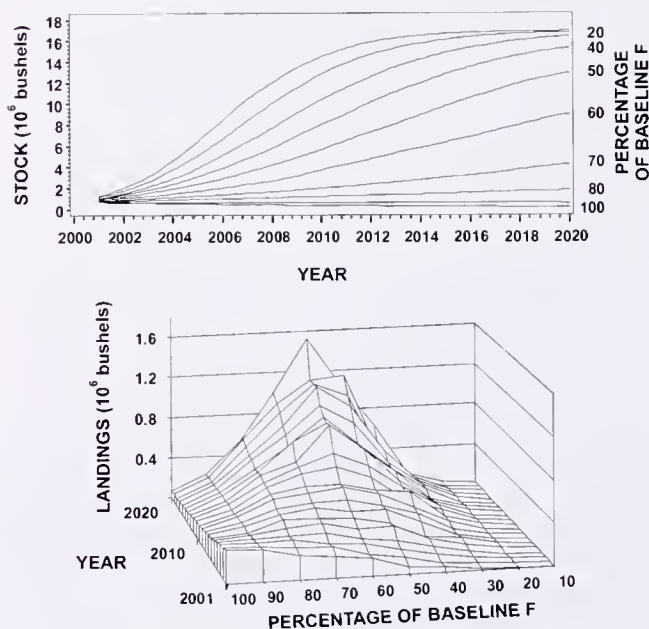


Figure 4. Annual mean simulated stock and landings for the bay-wide model at 10–100% of baseline F . Stock results were smoothed by polynomial spline interpolation; landings results were not smoothed.

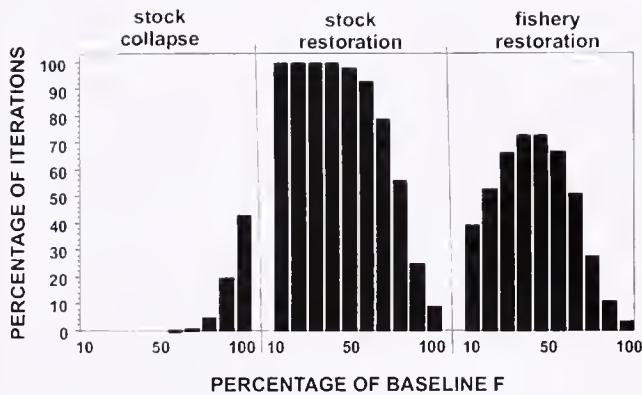


Figure 5. Percentages of iterations of the bay-wide model at 10–100% of baseline F that indicated stock collapse (market stock $< 0.1 \times 10^6$ bushels), stock restoration (market oyster stock $\geq 1.77 \times 10^6$ bushels), or fishery restoration (simulated landings $\geq 2 \times 10^6$ bushels). There were 1000 iterations for each value of F .

landings after an initial lag required for spat to reach market size (Fig. 6). A sanctuary scenario (i.e., coupling 1.1R with 0.9F) predicted approximately 3-fold increases in stock and landings within 20 y (Fig. 7). This scenario indicated a low likelihood (percentage of iterations) of stock collapse and moderate likelihoods of stock and fishery restoration (Fig. 8).

Hindcasts for all three salinity zones indicated exponential stock declines during the period 1986–2000 (Figs. 9–11). Simulated landings for the period were roughly consistent with reported landings for the low- and high-salinity zones, but less so for the medium-salinity zone. Forward simulations for the low-salinity zone indicated that F would have to be reduced to 0.6F to achieve increasing stocks of market oysters (Fig. 12). Long-term landings in this zone were stable at 0.8F and optimal at 0.6F (lower plot of Fig. 12). In the medium-salinity zone, stock increased at 0.7F and approached carrying capacity at 0.3F or less (Fig. 13). Long-term landings in the medium-salinity zone were stable at 0.9F and optimal at 0.7F (lower plot of Fig. 13). Moderate reductions in F had little or no effect on stock or landings in the high-salinity zone (Fig. 14). The model predicted substantially increasing stocks at 0.2F and 0.1F in this zone. Simulated long-term landings in the high-salinity zone attained maximum values at 0.1F, reaching 0.12×10^6 bushels by 2020, about 80% of 1986 landings from this zone. Simulations of a sanctuary strategy (0.9F, 1.1R) slowed, but did not reverse, stock declines in the low- and high-salinity zones.

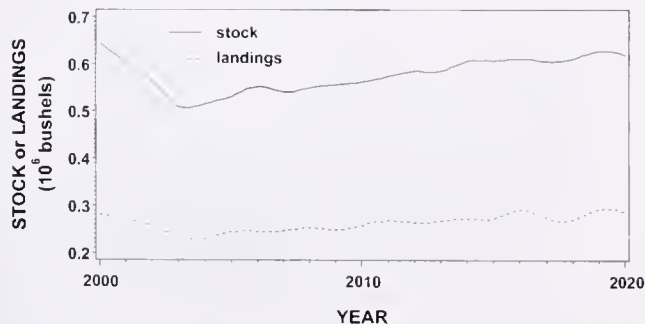


Figure 6. Annual mean simulated stock and landings for the bay-wide model with a 10% increase in R above baseline. Reported annual landings are shown on the lower plot; all values were smoothed by polynomial spline interpolation.

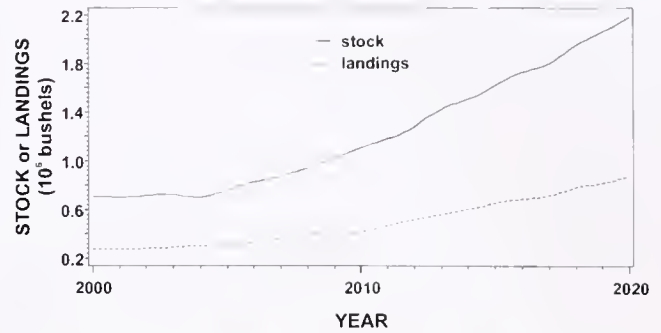


Figure 7. Annual mean simulated stock and landings for the bay-wide model with a 10% increase in R and 10% decrease in F from baseline values; smoothed by polynomial spline interpolation.

In the medium-salinity zone, the sanctuary simulation predicted an increasing stock after an initial lag (Fig. 15).

The stock restoration target for the low-salinity zone was an increase from 0.16×10^6 to 1.6×10^6 bushels by 2020. A high likelihood of achieving this target required 0.4F or less (Fig. 16).

DISCUSSION

We have quantified the population dynamics of the Maryland harvestable oyster stock and simulated the long-term consequences

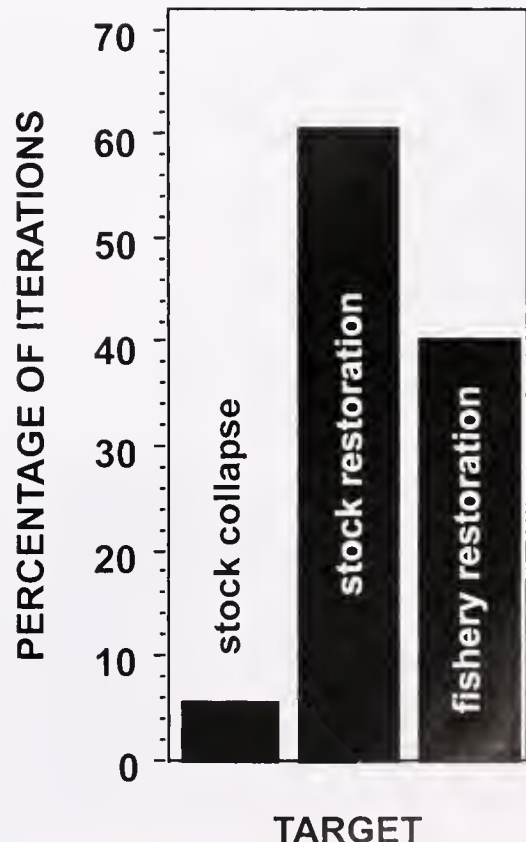


Figure 8. Percentages of 1000 iterations of the bay-wide model that indicated stock collapse (market stock $< 0.1 \times 10^6$ bushels), stock restoration (market oyster stock $\geq 1.77 \times 10^6$ bushels), or fishery restoration (simulated landings $\geq 2 \times 10^6$ bushels) with 10% increase in R and 10% decrease in F from baseline values.

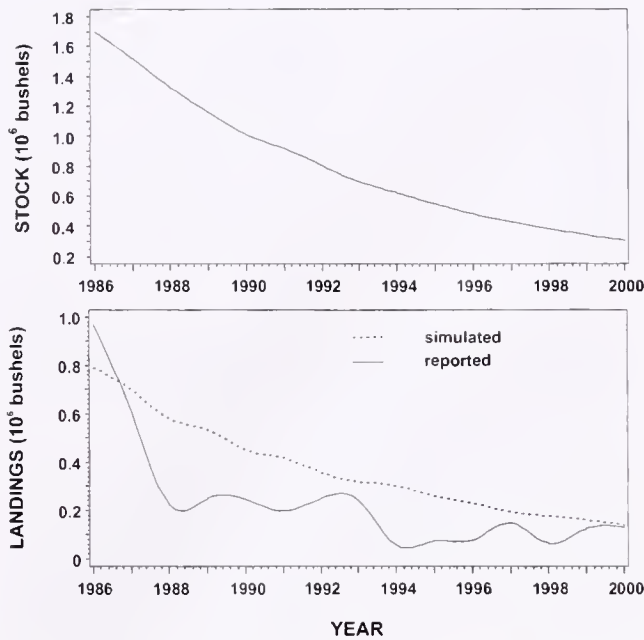


Figure 9. Annual means of simulated stock and landings from a hindcast of the low-salinity (<12 ppt) zone model. Annual reported landings are shown on the lower plot; no model-independent estimates of stock size were available for comparison. All values were smoothed by polynomial spline interpolation.

of several management scenarios for the stock as a whole, and also for three subpopulations segregated by salinity. The results indicate that there is potential for restoring the stock and the fishery to sustainable historical levels with reasonable management interventions. The following discussion elaborates on the uncertainties in

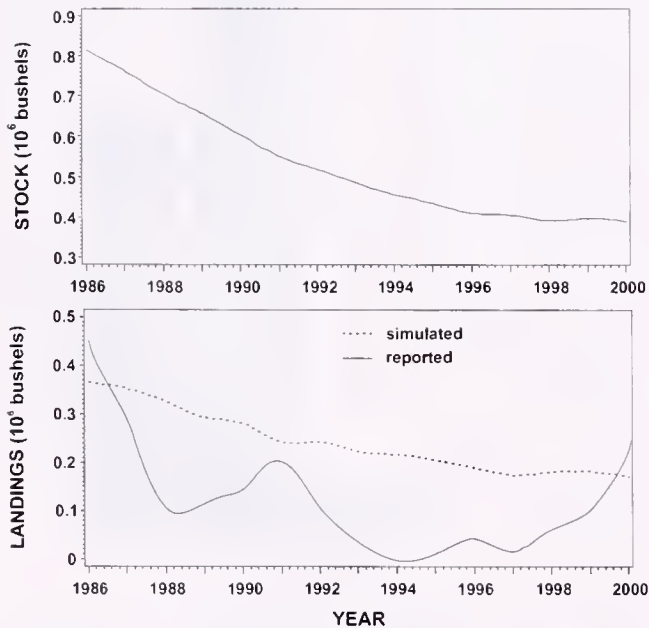


Figure 10. Annual means of simulated stock and landings from a hindcast of the medium-salinity (12–14 ppt) zone model. Annual reported landings are shown on the lower plot; no model-independent estimates of stock size were available for comparison. All values were smoothed by polynomial spline interpolation.

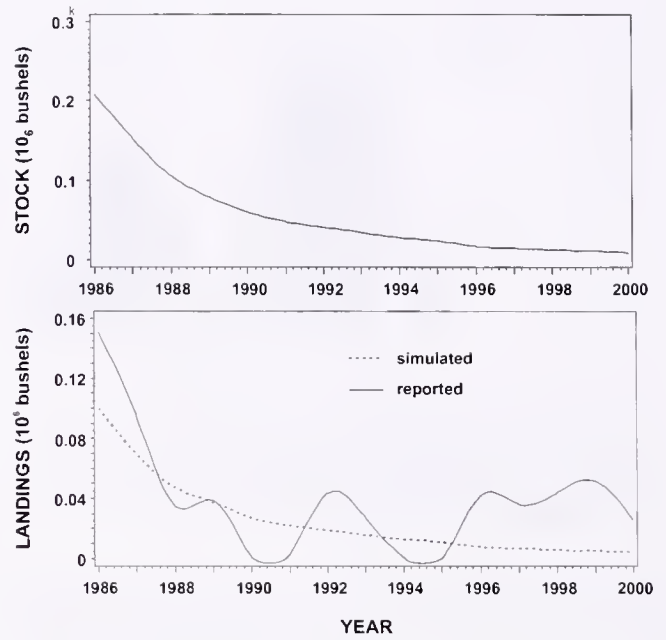


Figure 11. Annual means of simulated stock and landings from a hindcast of the high-salinity (>14 ppt) zone model. Annual reported landings are shown on the lower plot; no model-independent estimates of stock size were available for comparison. All values were smoothed by polynomial spline interpolation.

the data and assumptions that were used in the modeling process, examines management implications both prospectively and retrospectively, and offers recommendations for restoring sustainable oyster stocks in Maryland.

Uncertainties

There are at least three types of uncertainty associated with each of the model parameters: natural variation, measurement er-

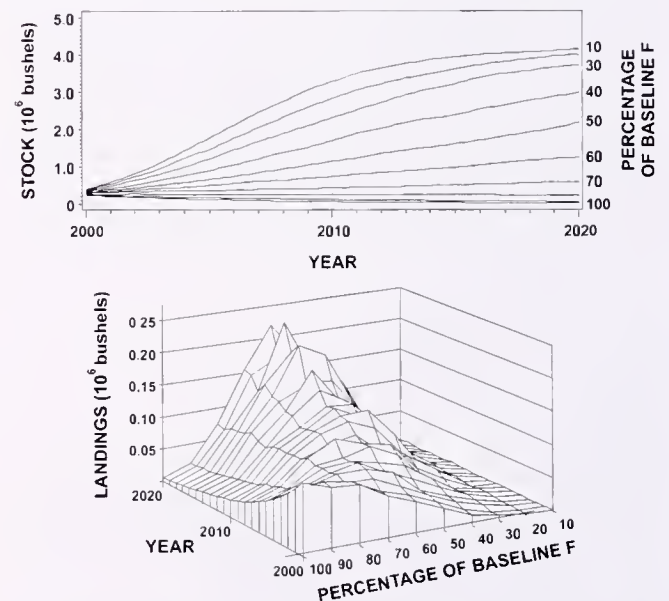


Figure 12. Annual mean simulated stock and landings for the low-salinity (<12 ppt) model at 10–100% of baseline F. Stock results were smoothed by polynomial spline interpolation; landings results were not smoothed.

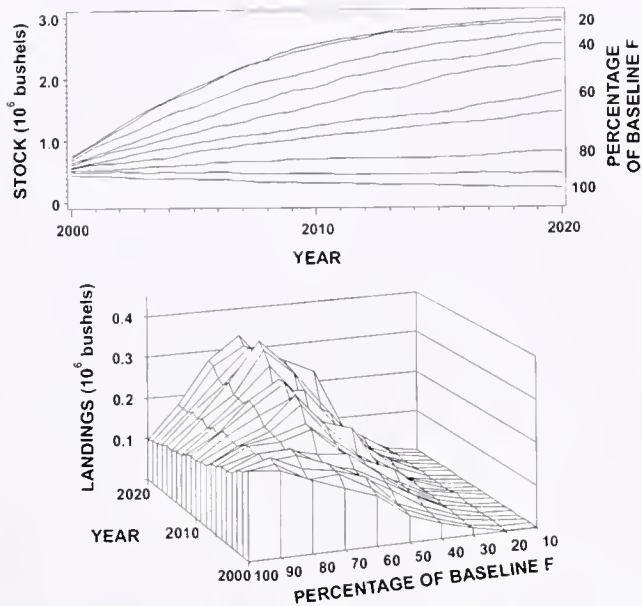


Figure 13. Annual mean simulated stock and landings for the medium-salinity (12–14 ppt) model at 10–100% of baseline F. Stock results were smoothed by polynomial spline interpolation; landings results were not smoothed.

ror, and inaccuracy (i.e., how closely sampling and estimation measure the true parameters). Natural variation is included in the model structure by allowing F, M, and R to vary according to observed temporal and spatial variation, and is applied to model results as shown in Figures 3 and 9. This variability permits a wide range of possible outcomes, for which we have assumed that log-normal mean trends represent the most likely. Measurement errors for the survey data and indices of relative abundance used to

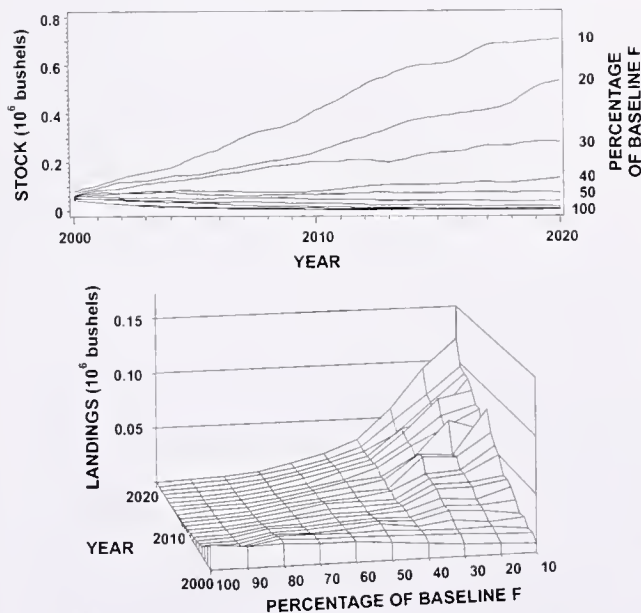


Figure 14. Annual mean simulated stock and landings for the high-salinity (>14 ppt) model at 10–100% of baseline F. Stock results were smoothed by polynomial spline interpolation; landings results were not smoothed.

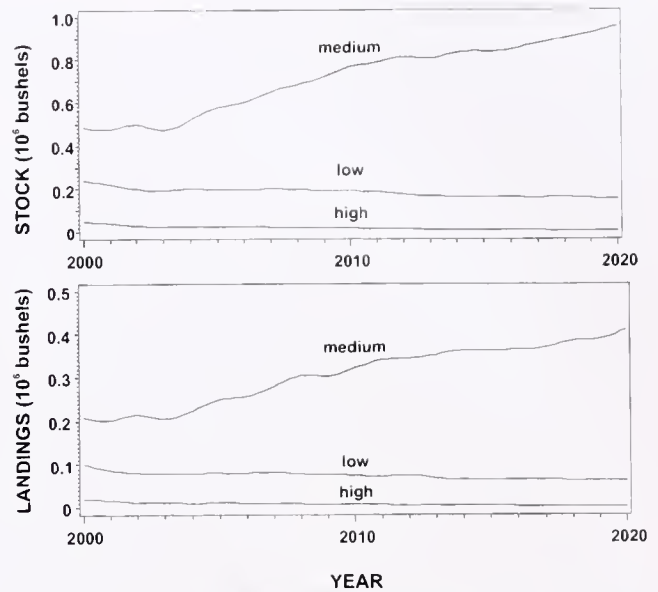


Figure 15. Annual means of simulated stock and landings for three salinity zones with a 10% increase in R and a 10% decrease in F from baseline values; all values were smoothed by polynomial spline interpolation.

parameterize the model have been shown to be small relative to temporal and spatial variations in the oyster population (Jordan et al. 2002, Jordan 1995), and will not be considered further here.

Parameter values are accurate only to the extent that the assumptions made in estimating them are valid. The use of box counts to estimate annual natural mortality has been a subject of investigation (Christmas et al. 1997, Powell et al. 2001, Powell et al. 2002) and was discussed in the context of the current study by Jordan et al. (2002). We conclude that because annual natural mortality estimated from box counts was strongly correlated with trends in the live oyster population (market oyster biomass; $r = -0.60$, $P = 0.01$) and landings ($r = -0.58$, $P = 0.02$), the method is sufficiently accurate.

The estimates of fishing mortality are based on an assumption tested only by the comparison of hindcast results to landings data

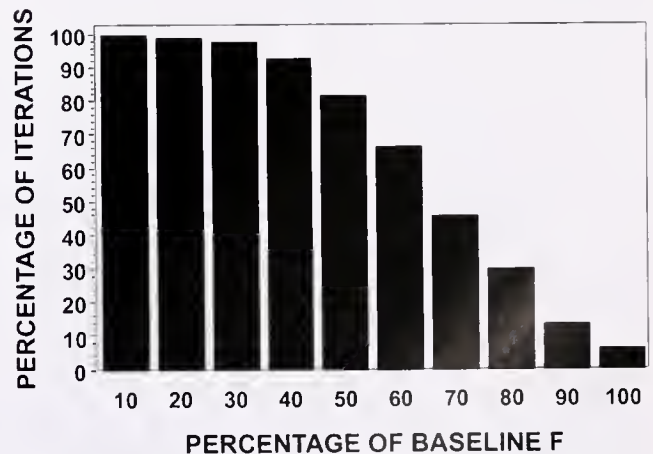


Figure 16. Percentages of iterations of the low-salinity (<12 ppt) model that indicated stock restoration (market stock $\geq 1.1 \times 10^6$ bushels) at 10–100% of baseline F.

(Fig. 2) and by the generally reasonable nature of simulations. Fishing mortality rates reported from prior stock assessments bracket our mean estimate ($F = 0.71$) for 1986–2001. Cabraal (1978) reported $F = 0.27$ from analysis of catch and effort data for 1975–1976, with a market oyster stock of 10.4×10^6 bushels and landings of 2.5×10^6 bushels. Rothschild et al. (1994) calculated $F = 1.3$ for the 1991 Maryland oyster harvest; we used Smith and Jordan's (1993) finite exploitation rate of 0.53 ($F = 0.64$) for the same year to scale F from relative to absolute values. Jordan et al. (2002) showed that the Rothschild et al. (1994) estimate was probably too high because of errors in specifying natural mortality and growth. Because this model is sensitive to F , a focus of this study, the estimates need better verification. The methods used to harvest oysters in Maryland are inherently inefficient (except for diving, a minor component of the fishery), thus very high values of F probably would require more fishing effort than would be returned by the value of the catch. High rates of effort might occur locally, or for brief episodes (viz. $F = 1.29$ for 1989), but should not be sustained.

The two mortality terms in the model, M and F , are strictly additive and do not interact. A model by Klinck et al. (2001) of the New Jersey oyster fishery in Delaware Bay predicted higher yields by adjusting fishing seasons to compensate for high rates of disease-related natural mortality. In effect, natural mortality could be decreased by concentrating the fishery in late spring, earlier in the year than most of the disease-related mortality. Moreover, much of the recruitment to market stocks in mid-Atlantic estuaries probably occurs in spring (April–May), when oyster growth rates tend to be high. For these reasons, stock size should peak in late spring. Klinck et al. (2001) used a daily time step and separate functional forms for M and F in their model. Unlike the New Jersey fishery, where a late spring–early summer harvest is traditional, the Maryland fishery has operated only in the fall and winter (October through March, with minor exceptions) for many decades. Although it is likely that higher yields could be achieved in Maryland at a given F with seasonal management, tactical fisheries management has not been the objective of this study. We have focused on long-term population restoration, for which an annual time step and additive mortality terms appear to suffice. Total annual mortality rate ($Z = M + F$) would not be affected by the compensatory mortality demonstrated by Klinck et al. (2001). From a long-term population or restoration perspective, it matters not how an oyster dies, only that it does.

We did not measure or validate directly the recruitment term (R) in the model; it was derived from year-to-year differences in the market stock, plus total mortality. Net recruitment [$dS/dt = r = R - (M + F)$], the rate of change in stock size, was consistent with observed long-term trends in relative stock biomass and reported landings. Gross recruitment rates were higher at higher salinity (Table 1), as expected because of higher spat settlement densities at higher salinity (Jordan 1995).

The estimates of carrying capacity (K), although based on the best available data, are uncertain and not directly verifiable. Fortunately, model results and conclusions are not especially sensitive to K , but low-mortality simulations for which the stock approaches K should be interpreted more cautiously than others. Our only verification of this parameter is that K for the bay-wide model ($\sim 17 \times 10^6$ bushels) is reasonable with respect to historical landings, which peaked at 10 to 15×10^6 bushels in the 1880s. Although K is a constant in this model, it certainly varies in the real world. Long-term declines in the quantity and quality of oyster

habitat (factors of K) in Maryland have been reported (Rothschild et al. 1994, Smith et al. 2001).

Besides uncertainties in estimating model parameters, the governing logistic equation may not accurately represent oyster population dynamics. A particular concern is the stock-recruitment (S/R) relationship. In this model, the rates of gross recruitment (R) and net recruitment (r) are not correlated with stock size, but the average absolute number of recruits increases linearly as the stock increases. The logistic formulation we use artificially limits stock size as a function of K without directly affecting the randomly input recruitment and mortality rates, in which density-dependence would be expressed in nature. Observations in Maryland have shown that oysters can produce high densities of spat at very low population levels; for example, two of the highest spat density indices on record were in 1991 and 1997, when adult population densities were very low (Maryland DNR 2001). This fact suggests that the S/R curve could be steep at low stock sizes, and if so, the model is conservative with respect to recruitment in these cases. Conversely, two major sources of natural mortality, diseases and predation, could be more intense at higher oyster densities, suggesting that the model could overestimate recruitment at moderate stock size; that is, the S/R curve could be flatter at moderate stock size than predicted by the logistic model (at large stock sizes, approaching K , errors in R would make little difference). Estimated stock size varied from 0.18×10^6 to 3.21×10^6 bushels (1–19% of K) during the time series of data used to parameterize the model, so we have observed only a minor portion of the S/R relationship, if indeed there is one. During this period, gross annual recruitment (R) ranged from 40% to 80% of the stock, without any obvious pattern. We quote Cushing (1968, p. 124): "... there is very often no relation between parent stock and subsequent recruitment at those levels of stock which support fisheries."

Implications and Recommendations for Management

The model indicates that long-term average recruitment rates should be ample to replace or rebuild Maryland oyster stocks if mortality rates could be reduced sufficiently. Natural mortality cannot be managed to a significant degree so long as parasitic diseases kill large proportions of oysters. If the population is developing disease resistance through natural selection, the process has been too slow to observe. Selective breeding and introduction of disease-tolerant oyster strains for restoration purposes have not been implemented at a scale that could make more than a local difference. Attempts to improve management by restricting movements of infected seed oysters have had no measurable effects at the population scale.

Fishing mortality, in principle, is entirely controllable. The Maryland oyster fishery is regulated by gear restrictions, seasonal and area closures, a minimum size limit, daily catch limits, license surcharges, and limited entry. These restrictions, most of which pre-date the major impacts of Dermo and MSX diseases, apparently have not been sufficient to maintain sustainable rates of fishing mortality while natural mortality rates are high. Annual indices of relative and absolute stock size (Jordan et al. 2002) are available that could be used to limit fishing mortality by setting a total allowable catch (TAC), based on a conservative F , for each year. The fishery could then be closed whenever in the season the TAC was attained, or effort could be otherwise limited so as not to exceed the TAC. The Maryland bay-wide model indicates that reducing average F by 40% ($F = 0.43$) to 50% ($F = 0.35$) would

virtually assure stock restoration and an enhanced fishery within a decade.

As an example, the estimated initial stock for the 2000–2001 oyster season was 702,000 bushels (Table 1), which produced landings of 348,000 bushels ($F = 0.70$, near the long-term mean). At $0.4F$ ($F = 0.43$), landings would have been about 245,000 bushels, and at $0.5F$ ($F = 0.35$), about 207,000 bushels. These losses to the fishery would be economically significant (\$2–4 million in dockside value) in the short-term, but more than compensated in the long-term. With $F = 0.43$, the model predicts average landings of 656,000 bushels for 2001–2020 and average landings $>1 \times 10^6$ bushels in the second decade of the simulation.

Model scenarios indicated little likelihood of rebuilding the stock or increasing landings by managing fishing mortality in the high-salinity zone. Maryland uses higher salinity areas, where spat settlement is relatively high, for seed oyster production. Large plots are shelled each summer to stimulate spat settlement. The following spring, the spat and shell are transferred to lower salinity areas where disease-related mortality is less. In effect, this strategy enhances recruitment in low-salinity areas, where natural recruitment is undependable. The bay-wide and low-salinity versions of the model implicitly include this source of recruitment, because some of the low-salinity monitoring sites were supplied with transferred seed oysters in most of the baseline years of this study. Because it is unlikely that a significant harvestable stock can be restored in the high-salinity zone, seed oyster production for supplementation of low-salinity areas is probably the best management strategy for this zone. There are benefits to stimulating recruitment through hatchery spat production, but cost and logistical constraints should be expected to limit this contribution to roughly the 10% increase in recruitment we have simulated (Figs. 6, 7, and 15).

Model predictions are most promising for the medium-salinity zone. This large area benefits from moderate to high spat settlement in some years and also from episodes of low salinity that can diminish disease-related mortality for one or more years. The model indicates that a sanctuary strategy alone could have modest success in this zone, in contrast to the low- and high-salinity zones. Management for this zone could use sanctuaries, closed to harvest and stocked with seed oysters, in combination with explicit reductions in fishing mortality, to rebuild productive stocks.

A productive oyster fishery was sustained in Maryland until repeated disease epizootics drastically increased natural mortality

rates beginning in the 1980s. Fishing mortality rates, meanwhile, remained constant or increased. Recruitment, whether stable or declining, could no longer keep pace with total mortality rates and the stock declined. This dynamic has continued into the 2000s. Strategies intended to increase recruitment (habitat improvement, natural and hatchery seed oyster production) have been used extensively, but they are expensive, have not stemmed the decline, and offer only marginal hope for the future.

Preliminary landings for the 2002–2003 Maryland oyster season are 51,145 bushels, about 2% of 1975 landings and 3% of 1986 landings. At a dockside value of no more than \$1.5 million, the fishery is close to economic extinction. We recommend that it is past time to reduce and control fishing mortality specifically to restore the oyster stock. With F set at 0.43 ± 0.25 (standard deviation) in 1986 and maintained at that level, mean simulated 2002–2003 landings would have been 1.96×10^6 bushels, 38 times the reported quantity. Ecological contributions (Jordan 1987, Newell 1988, CBP 2002) would have multiplied by roughly the same factor. Taking this action in 1986 would have caused a loss of nearly \$9 million in dockside value to the fishery for that year, but the loss would have been repaid many times in sustainable harvests and ecological services.

ACKNOWLEDGMENTS

We greatly appreciate assistance, advice, and support from staff of the Maryland Fisheries Service and Sarbanes Cooperative Oxford Laboratory, especially Mark Homer, Kelly Greenhawk, Gary Smith, Mitchell Tarnowski, and Jim Uphoff. This model was made possible by the long-term data collection efforts of many people from the Maryland Department of Natural Resources. William Fisher provided a thoughtful preliminary review of the manuscript. Financial support was provided by the Maryland Department of Natural Resources, the National Oceanic and Atmospheric Administration Chesapeake Bay Office, and the U.S. Environmental Protection Agency Chesapeake Bay Program. The information in this document has been funded in part by the U.S. Environmental Protection Agency. It has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. This is contribution number 1185 of the Gulf Ecology Division.

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AGE STRUCTURE AND GROWTH IN AN UNUSUAL POPULATION OF PURPLE CLAMS, *AMIAANTIS PURPURATUS* (LAMARCK, 1818) (BIVALVIA; VENERIDAE), FROM ARGENTINE PATAGONIA

ENRIQUE M. MORSÁN¹ AND J. M. (LOBO) ORENSANZ²

¹Instituto de Biología Marina y Pesquera "Almirante Storni," Universidad Nacional del Comahue and Dirección de Pesca de Río Negro, P.O.Box 104, (8520) San Antonio Oeste, Argentina; ²Centro Nacional Patagónico (CONICET), (9120) Puerto Madryn, Argentina

ABSTRACT The purple clam, *Amiantis purpuratus*, is distributed in the southwest Atlantic between Vitoria (Brazil, 20° SL) and San Matías Gulf (Argentina, 41° SL). We studied the age structure and growth in its southernmost population, which sustains small artisanal and sport fisheries. Examination of individuals collected in 1995 revealed well-marked external growth bands on the shells and a puzzling pattern: most had 15 or 16 bands. Were these annual, the implication would be virtually no successful recruitment during the 14-year period 1981–1994. We recovered and processed samples collected in the same area in 1980–1983 and 1987–1990 and conducted a comprehensive survey of the population in 1995. Examination of thin shell sections showed an almost exact correspondence between internal and external bands. The time series of size-at-age data indicated a bi-univocal correspondence between years elapsed and number of bands accumulated. This confirmed that external bands are annual and that only two significant year-classes (1979 and 1980) settled in the study area over a period of at least 15 years (1979–1994). Growth was studied at one location using size-frequency distributions and size-at-age data. Comparisons were made using likelihood methods to test differences among year-classes (1979, 1980, older) and between two sites. Differences between the 1979 and 1980 year-classes were insignificant at both sites. Growth rate estimated for a pool of pre-1979 year-classes was high when compared with the 1979–1980 year-classes. Density dependence is advanced as a hypothesis to explain the comparatively slow growth of the 1979–1980 year-classes. Longevity is above 25 years. Implications of the findings for conservation and management are discussed.

KEY WORDS: *Amiantis*, purple clam, growth, age, recruitment, southwest Atlantic

INTRODUCTION

The purple clam, *Amiantis purpuratus* (Lamarck, 1818), inhabits intertidal and shallow subtidal sandy bottoms along the Atlantic coast of South America, from 20°S to 41°S (Scarabino, 1977; Rios, 1994) (Fig. 1A). The southernmost population within this broad latitudinal range, confined to the northwest of San Matías Gulf (Fig. 1B), appears to be relatively isolated. This is, also, the only stock of the species subject to exploitation. What started as a recreational intertidal fishery in the area of Playa Villarino (Fig. 1C) has evolved since 1996 into a small-scale commercial diving fishery. Annual catch peaked at 382 t in 1996, and since then has fluctuated around 100 t. A preliminary survey conducted in 1995 revealed that (i) there were no juveniles, (ii) clam shells had very conspicuous external bands, and (iii) most shells had 15 or 16 bands. If those bands were laid annually, these preliminary observations would imply that the purple clam is a long-lived species, that the incipient fishery was based on two year-classes, and that pulses of recruitment are very sporadic. These prospects raised concerns with regard to the conservation of this fringe population and the sustainability of the fishery that it supports. Because there were no published antecedents on purple clam biology and ecology, we initiated a study to investigate the dynamics of this population. The first step was to develop reliable aging techniques. The simplest method traditionally used to age bivalves is based on the interpretation of external growth rings on the surface of the shells, often formed during the winter in cold-temperate seas (Richardson and Walker, 1991). In many species, however, such rings are absent or are difficult to interpret (Richardson et al. 1993). An alternative method is the study of the optical pattern of internal bands in shell cross-sections, using either acetate peels or thin sections (Cerrato 2000). Internal growth bands are more clearly defined and easier to count in shell structures such as hinge plates and chondrophores (Palacios et al. 1994; Thompson et al. 1980).

The external growth rings of purple clam shells are very conspicuous, with an alternation of thick dark purple and thin pink bands. Here we report results on the nature of internal growth bands, on the matching between external rings and internal bands, and on their periodicity. Based on age data and on the analysis of size-frequency distributions, we estimated growth parameters, compared growth curves between cohorts and between sites in the study area, and advanced hypotheses on the causes of the variation observed. This is the first study of this type conducted for this potentially significant shellfish resource. Implications of our results for the dynamics of the population and the management of the fishery are discussed.

MATERIALS AND METHODS

Study Area

Playa Villarino (Fig. 1C) is a 9-km long dissipative sandy beach located in the proximity of San Antonio Bay (NW of San Matías Gulf). Average tidal amplitude is 7.62 m (maximum 9.2 m); in low tide the beach is 450–600 m wide. Sediment is predominantly fine sand, with patches of shell hash. Water temperature ranges, on average, from 6°C in August (winter) to 22.5°C in January (summer) (Kroeck and Morsán 1995). Water circulation in the NW of San Matías Gulf was described by Lanfredi and Pousa (1988) as a clockwise coastal eddy predominantly influenced by tidal currents, with limited interaction with the general circulation in the gulf. The population under study is distributed in the intertidal and subtidal zones, down to 10 m depth in low tide. The macrofauna is dominated by the purple clam (average biomass: 3235 g m⁻² in a survey conducted in 1995; Morsán, 2000), followed by two scavenging/carnivore gastropods: *Buccina-nops gobulosum* (NASSARHIDAE) and *Olivancillaria urceus* (OLIVIDAE).

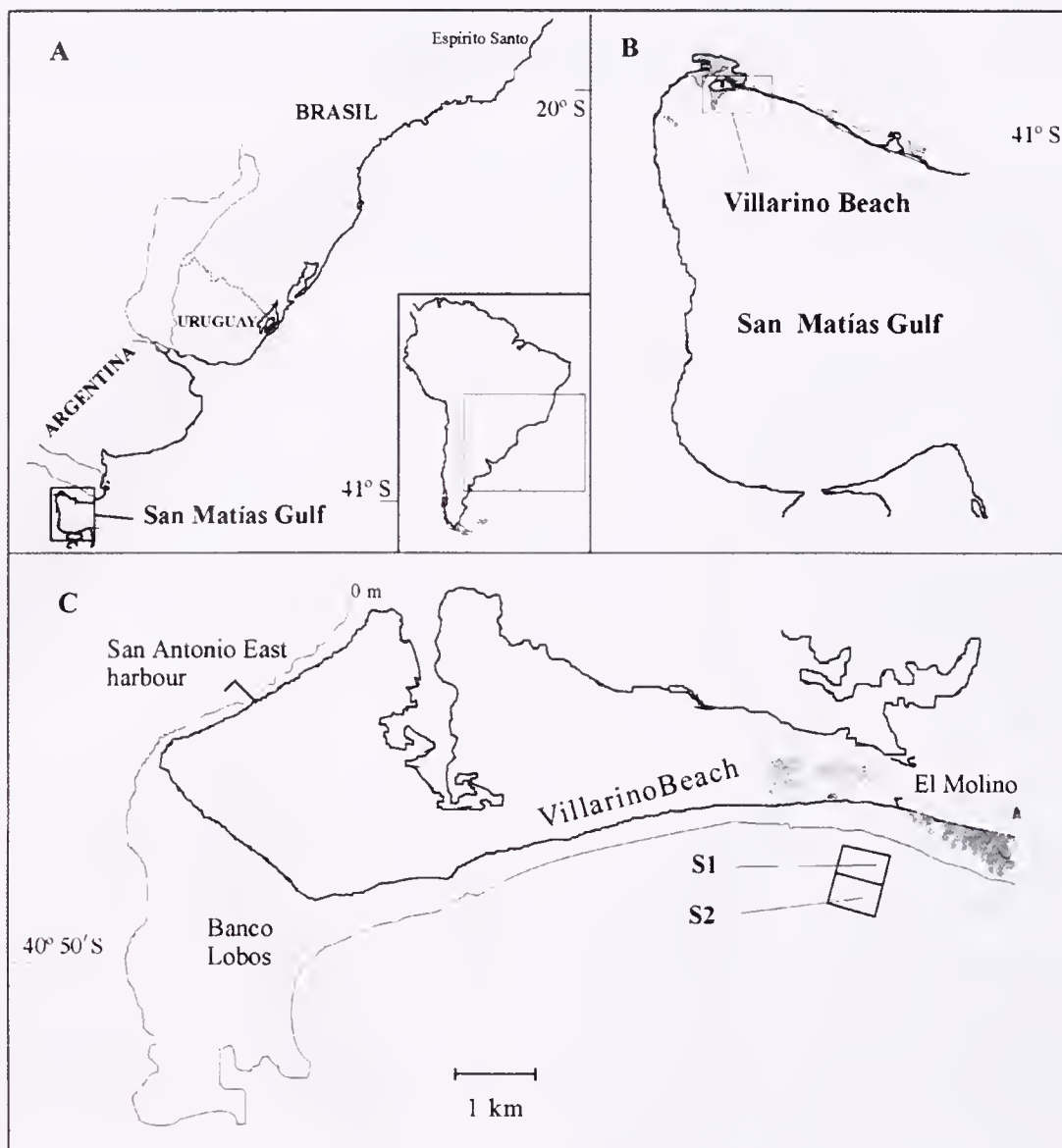


Figure 1. (A) Geographic distribution range of the purple clam, *Amiantis purpuratus*. (B) San Matías Gulf and Villarino Beach. (C) Location of sampling sites at El Molino (S_1 and S_2).

Study Site

Studies on age and growth were conducted at El Molino (Fig. 1C), a site located near the eastern end of the beach where periodic samples were obtained within a rectangular plot ($500 \text{ m} \times 1000 \text{ m}$) perpendicular to the shoreline. Depth varies from 3 m to 9 m; strata were defined as the shallow (average depth 3.2 m) and deep (average depth 7.7 m) halves of the plot, respectively designated as sites S_1 and S_2 . Maximum density during the 1995 survey was 368 clams m^{-2} in S_1 and 216 clams m^{-2} in S_2 .

Sampling Protocol

Samples were obtained by divers between 1980 and 1995 (Table 1). Sampling units consisted of 0.25 m^{-2} quadrats dug to a depth of 20 cm. On a few occasions, samples were qualitative although obtained in a nonselective way. Between 1980 and 1983

clams were dug manually; an airlift was used starting in 1987. Total height (maximum distance from umbo to ventral margin) was recorded for all clams. The samples were obtained during three time intervals, under different arrangements (Table 1):

- (1) 1980–1983: These samples, obtained before the beginning of our project, were believed to be lost. We reconstructed their fate, tracked their location, and finally recovered them through a sequence of serendipitous circumstances. They proved extremely valuable. The data include date, position, number of individuals, and individual sizes for 15 samples, and two sets of valves collected in 1982. The shells of individuals collected in October 8, 1982, that had presumably settled before 1979 (based on external bands, $n = 104$) were selected for aging.
- (2) 1987–1990: Periodic sampling. One sample was used for age determination ($n = 55$).

TABLE 1.

Samples collected between 1980 and 1995. S_1 and S_2 are sample sites, respectively shallow and deep strata of the El Molino plot. N/A: nonquantitative, nonselective samples.

Sampling Period	Date (month/day/year)	Site	Quadrats	Number of Individuals in Sample	Number of Individuals Aged
1980–1983	11/12/1980	S_1	N/A	107	
	1/3/1981	S_1	N/A	98	
	12/7/1981	S_1	N/A	404	
	1/19/1982	S_1	2	275	
	2/1/1982	S_1	2	233	163 ¹
	3/19/1982	S_1	3	1370	
	8/27/1982	S_2	3	601	
	10/8/1982	S_2	2	650	104 ²
	4/22/1983	S_1	1	199	
	7/8/1983	S_2	1	343	
	9/15/1983	S_2	1	368	
	10/26/1983	S_1	2	737	
	11/11/1983	S_2	2	333	
	12/2/1983	S_2	1	442	
	12/22/1983	S_2	3	676	
1987–1990 (periodic samples)	4/3/1987	S_1	N/A	413	
	10/5/1987	S_1, S_2	2	316	
	1/9/1988	S_1, S_2	2	167	
	2/29/1988	S_2	N/A	372	
	9/8/1988	S_2	N/A	352	
	2/16/1989	S_1	N/A	261	
	1/9/1990	S_1, S_2	1	261	
	1/22/1990	S_2	N/A	248	
	2/1/1990	S_1	N/A	320	55 ³
	3/23/1990	S_2	1	247	
	9/29/1990	S_1	N/A	164	
	11/28/1990	S_1	N/A	191	
1995 (survey)	3/10/1995	S_2	8	280	180 ⁴
	3/24/1995	S_1	4	208	160 ³
	5/27/1995	S_1, S_2	8	247	

¹ External bands counted but not measured.

² External bands counted and measured.

³ External bands counted but not measured.

⁴ External bands counted and measured; internal bands counted.

- (3) 1995: Quantitative survey of the entire population, conducted in March 1995. Individuals from two samples were used for age determination.

Analysis of SFDs

Size data were grouped at 1-mm intervals. Mean size, standard deviation, and proportion of each annual separable modal group were estimated for each sample unit. In some cases the groups could be identified visually. When modal groups overlapped, the decomposition was done by means of maximum likelihood methods, assuming that the components were normally distributed (MacDonald & Pitcher 1979).

Age Determination

Subsamples from five samples were used for aging (Table 1). Of these, two subsamples from the 1995 survey were used to study external and internal bands. External bands were counted and measured in a third subsample (sample collected on October 8, 1982), and only counted in two subsamples (samples collected on February 1, 1982, and February 1, 1990).

The external banding pattern of purple clam shells is very conspicuous, with an alternation of thick dark purple and thin pink bands. We counted the number of pink narrow bands; the beginning of the well-defined pink band was defined as the boundary between two consecutive bands. Bands were measured (distance from the umbo to the distal border of the band) along the same axis used to measure total height and to cut the shells for the observation of internal bands (see below).

To count internal bands, thin sections were obtained, cutting each valve with a low-speed diamond saw along the same axis used to count and measure external bands, running from the umbo to the ventral margin through the hinge plate. The surface left by the cut of one of the two valve halves was ground and polished using sandpaper of very fine grain (4000 grit) on a rotating platform at variable speed. The polished surface was glued with epoxy resin to a microscope slide. A 0.5-mm thick slice was obtained by means of a second cut along a plane parallel to the slide's surface. The exposed face of the thin section was ground and polished with sandpaper of medium grain (1000 grit) and very fine grain (4000 grit) until adequate thickness and texture were reached. Mounted thin sections were examined under a dissecting scope with trans-

mitted light to identify internal growth bands and to establish their correspondence with external bands.

Growth Modeling

Growth was modeled using (i) size-frequency distributions (SFDs) obtained between 1980 and 1995 and (ii) size-at-age back-calculated using external band measurements (H_i , where i indicates the boundary sequential number counting from the umbo) in samples collected in 1982 and 1995 (Table 1). Likelihood methods were used to fit the von Bertalanffy growth model,

$$H_t = H_{\infty} (1 - e^{-k(t-t_0)}) + \varepsilon,$$

where H_{∞} is asymptotic shell height (expressed in mm), k is a constant (expressed in yr^{-1}), t is age (in years), t_0 is the age at size zero and ε is an error term distributed normally ($\varepsilon \sim N(0, \sigma)$). In doing so, we assumed that external bands are laid down annually (see Results for validation of this assumption). The likelihood ratio test was used to compare growth curves between cohorts and/or locations (Kimura 1980; Cerrato 1990). Null hypotheses are of the form

$$H_0: H_{\infty,1} = H_{\infty,2}, \dots; k_1 = k_2, \dots; t_{0,1} = t_{0,2}$$

and combinations of them, where 1 and 2 denote the two statistical populations being compared. Under the null hypothesis, the test statistic $-2\log(\Lambda)$ converges asymptotically to a $\chi^2_{(g)}$ distribution with g degrees of freedom (equal to the number of fixed parameters).

RESULTS

Age Determination

Matching between external and internal growth bands (Fig. 2) was almost perfect. Internal growth lines are more evident in the hinge plate when the cut is perpendicular to the valve and runs across one of the cardinal teeth. Clams sampled during the late summer of 1982 ($n = 163$, Table 1) had two or three pink bands. Eight years later, in late summer 1990, individuals ($n = 58$) collected in the same location showed 10 or 11 bands. Finally, most individuals (97%) in two samples collected during late summer in 1995 ($n = 180$ and 160 , Table 1) had 15 or 16 bands (Fig. 3). This

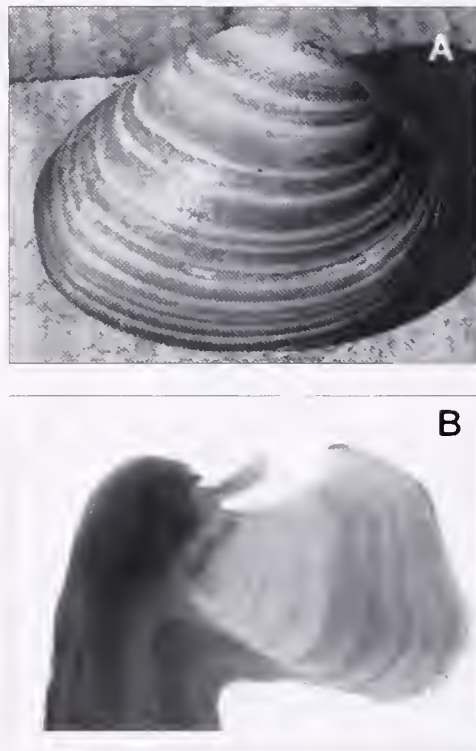


Figure 2. Growth pattern in the shell. (A) External view of a shell showing the external growth bands; (B) cross-section through the hinge region of a 13+ year-old individual.

indicates that between 1982 and 1995 the population was dominated by two year-classes and confirms that internal and external bands are laid annually. A dark purple band was visible along the border of the valves in all specimens collected in late summer. Summer is the spawning season of the purple clam in Villarino. Although the length of the larval stage is unknown, settlement is assumed to occur in late summer. Purple clams would mark their first clear growth band during the following winter. If this interpretation is correct, then the two cohorts must have settled during late summer in 1979 and 1980. The number of bands in shells from

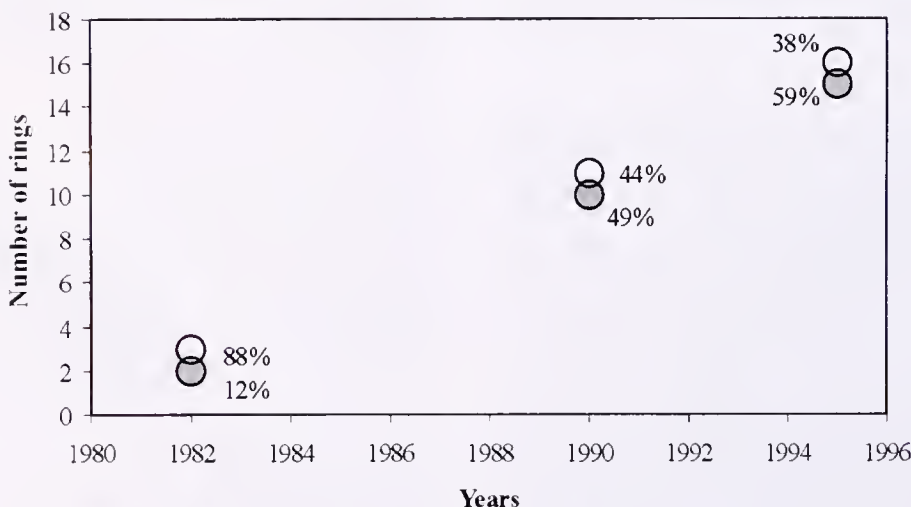


Figure 3. Correspondence between time elapsed between sampling dates and bands accumulated. Empty circles, 1979 cohort; solid circles, 1980 cohort. Percentages refer to number of individuals aged per sample.

old clams collected on 10/8/1982 (Table 1) varied from 9 to 18, indicating that they settled during the period 1965–1974. These cohorts had vanished from the population in 1995. The maximum number of bands recorded (i.e., maximum inferred longevity) was 42 years.

SFD Analysis

The progression of SFDs from 1980 to 1995 is shown in Figure 4 (seasonal pools, only six periods represented). Individuals smaller than 25 mm prevailed between 1980 and 1983, with the exception of a few larger than 40 mm. Between 1987 and 1990, most of the clams ranged between 25 and 35 mm. Finally, in 1995, most clams were larger than 35 mm. No juveniles were found after 1982. Two modal components were clearly distinguishable in the SFDs through 1987; afterward they overlap, making their recognition difficult. The presence of two consecutive cohorts in each of the subsamples used for aging (Table 2) is consistent with the two modal components observed in the SFDs, presumably corresponding to the year-classes settled in 1979 and 1980. The mean size of modal components estimated for all sampling units clearly shows the growth of these two cohorts between November 1980 and May 1995 (Fig. 5).

Growth

Growth rate was very similar between the 1979 and 1980 cohorts (Fig. 6). Although the complete parameter vectors were significantly different (H_04 , Table 2), pairs of analogous parameters do not show significant differences ($P > 0.05$, H_01 – H_03 , Table 3). Parameters H_{∞} and k were analyzed together in a single test (H_05). The null hypothesis was rejected only for estimates based on growth-band data. Differences between the 1979 and 1980 cohorts were not significant within each stratum, although differences between strata were significant for both cohorts (H_05 , Table 3). Parameter k did not differ significantly between groups in any case. Difference between sites was driven by parameter H_{∞} , which was higher in the deepest stratum where density was lower. Growth parameters estimated on the basis of size-at-age back-calculation for the multicohort group sampled in 1982 were higher than those of the 1979–1980 cohorts (Fig. 6). The null hypothesis of equal growth coefficients (k) could not be rejected, but all others were ($P < 0.01$).

DISCUSSION

When a preliminary survey of purple clams was conducted in Playa Villarino (San Matías Gulf, northern Argentine Patagonia) during the summer of 1995, some aspects of this population proved intriguing. First, there were no juveniles; second, most shells had 15 or 16 conspicuous external bands. If interpreted as annual, these preliminary observations would imply that the purple clam is a long-lived species, that the incipient fishery was based on two year-classes, and that pulses of recruitment are very sporadic. Correlating information obtained during three intervals over a period of 15 years (Table 1) confirmed that, indeed, the population had been dominated all along by two year-classes, presumably settled during the summers of 1979 and 1980. Three pieces of information are remarkably consistent with each other, substantiating that hypothesis: (i) the number of external bands increased bi-univocally with time over the years (Fig. 3), (ii) there was an almost perfect correspondence between external and internal bands (Fig. 2), and (iii) the progression of SFDs through time show a modal structure that

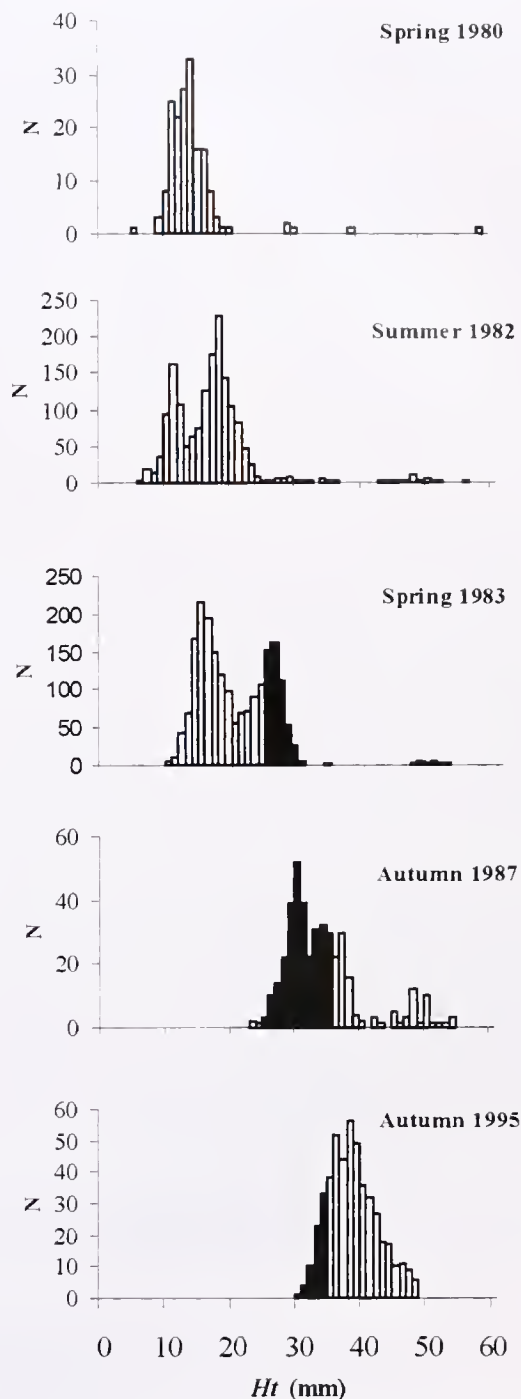


Figure 4. Size-frequency distributions (SFDs) during the period 1980–1995. Samples were pooled into several seasons. Solid bars indicate the range 25–35 mm for visual reference.

matches the age structure inferred from the analysis of growth rings (Fig. 4).

The recovery of data and samples collected in 1980–1983 (presumed lost) was most significant for the interpretation of the data. Shells collected at that early time in the life history of the two cohorts facilitated tracking the latter through time and validation of growth marks. External annual bands in this purple clam stock are among the clearest reported for bivalves, to the extent that we first thought of them as an endogenous sculpture pattern. This may be

TABLE 2.

Growth parameters estimated for different cohorts at El Molino, using size-at-age and size-frequency distribution (SFD) data.

Cohorts	Site	Size-at-age Data			SFD Data		
		k	H_{∞}	t_0	k	H_{∞}	t_0
1965–74	El Molino	0.154	57.2347	−0.246	—	—	—
1979		0.1329	43.545	−1.412	0.149	44.125	−0.424
1980		0.1333	42.025	−1.199	0.095	48.971	−0.863
1979	S_1	0.1340	40.533	−1.402	0.1758	40.925	−0.375
	S_2	0.1233	45.631	−1.820	0.1390	47.553	−0.030
1980	S_1	0.1385	40.947	−1.038	0.1185	43.649	−0.679
	S_2	0.1159	45.208	−1.820	0.1028	48.906	−0.399

due in part to the strong seasonal variation in temperature and in part to the fact that the porcelain-textured and color banding of the outer surface of the shells, which are rarely deteriorated, greatly facilitate observation.

The virtual absence of new settlers during the 15-year period 1981–1995 indicates that the population is sustained by very sporadic pulses of settlement/recruitment. This could be explained by two hypotheses. The first, and most obvious, is dependence of reproductive success on suitable combinations of environmental conditions (environmental windows) that occur only sporadically. This is to be expected: this being the southernmost population of the species,³ those windows may open up only occasionally. The dynamics of the purple clam may be representative of that of other species in the same region. Biogeographically, the northwestern corner of San Matías Gulf is an enclave where populations of several warm-temperate species live in apparent isolation, southward from their normal range of distribution. There is evidence of the recent local extinction of some of those, for example, the common drumfish (*Pogonias chromis*, Sciaenidae), whose otoliths are found in shell middens but which has not been recorded in the region during historical times. Sporadic pulses of recruitment may be indicative of the dynamics of extinction, perhaps the fate of this population unless there were a significant change in the environment (e.g., global warming) that reverses the trend.

³A few isolated individuals have been found further south, in San José Gulf (El Fracaso and El Riacho Beaches, JO, personal observation), but they do not seem to belong to a self-sustaining population. These are considered here as extralimital records.

A second plausible hypothesis that could explain sporadic settlement/recruitment is density dependence. Density and biomass observed during the 1995 survey (15 years after the settlement of 1979–1980 cohorts) were very high (Morsán, 2003), and rough back-calculations indicate that they must have been so over the preceding years. Compensatory density-dependent settlement in dense populations of suspension-feeding bivalves has been documented or hypothesized for other populations (Wilson 1991; Bachelet et al. 1992). If the density of residents were inhibiting the settlement/recruitment of new year-classes, the effects of density dependence should likely be observed in growth as well. Interestingly, individuals of the 1979–1980 year-classes reached comparatively small sizes-at-age. Asymptotic height estimated with different methods was in the range of 40–49 mm. By comparison, Carcelles (1944) reported much larger specimens (maximum height 72 mm) collected at the same location (Playa Villarino) during the period 1923–1933, close to maximum height reported for the species (73 mm, in subfossil deposits; Camacho 1966).

Size-at-age (Figs. 5 and 6) and estimated growth rate (overall sizes, Fig. 7) were much higher when estimated with data from a subsample of shells settled before 1979 (1965–1974), collected in October 1982. As compared with the 1979–1980 cohorts, asymptotic size was much higher in this multicohort group (57.2 mm) although differences in parameter k of the von Bertalanffy model were not significant (Table 3, Fig. 7). According to general theory (Sebens 1987) and empirical evidence (Guillou and Sauriau 1985; Harrington 1987), differences in k (which reflects catabolic activity) should be influenced by temperature, whereas variation in asymptotic size should reflect food availability. The latter should

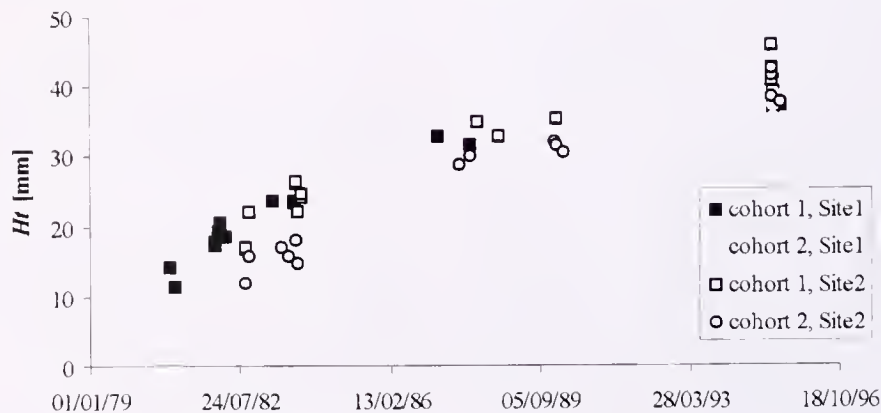


Figure 5. Temporal trend of mean size by cohorts and sites.

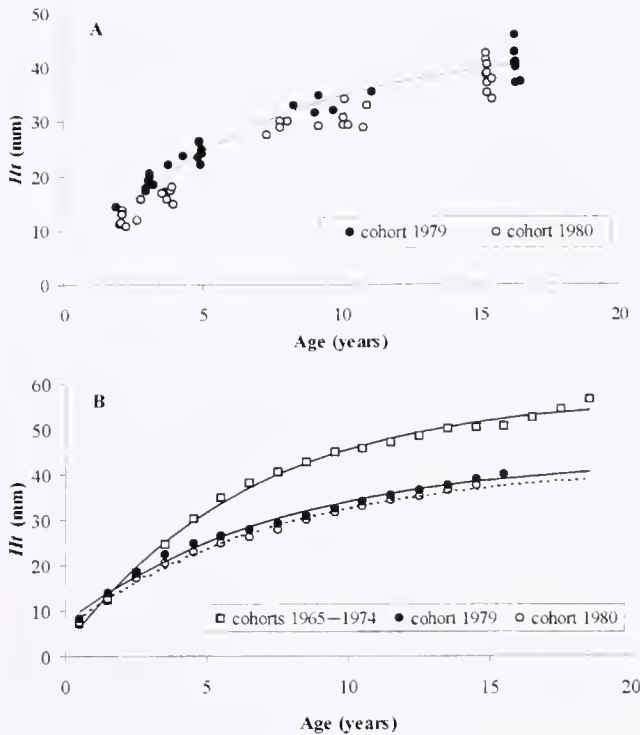


Figure 6. Estimated growth curves. (A) SFD data. (B) Back-calculated size-at-age data, multicohort group 1965–1974, and cohorts 1979 and 1980.

be affected by density, at least under high-density conditions such as observed during the survey when density reached 620 clam m^{-2} (corresponding to a biomass of 10 kg m^{-2} ; Morsán, 2003). Density-dependent growth in clams, long hypothesized and demonstrated experimentally under field conditions (e.g. Peterson 1982; Peterson and Black 1987), is proposed here as a hypothesis to explain the comparatively low growth rate of the 1979–1980 year-classes. It is interesting that although there was a big difference in asymptotic size, there was virtually no difference in parameter k , even when the pattern could have been concealed by structural correlation between the two parameters. This hypothesis will be explored in a forthcoming study.

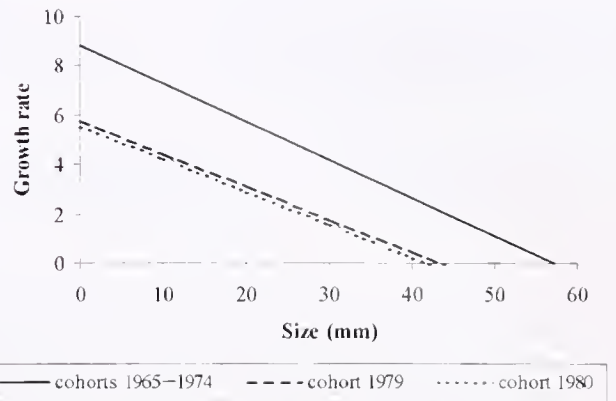


Figure 7. Estimated growth rate as a function of size. Cohorts compared, age data.

The oldest specimens belonging to pre-1979 year-classes collected in 1982 were 17 years old. The 1979–1980 year-classes were 16–17 years old when the 1995 survey was conducted. Recent informal observations by one of us (EM, 2003) indicate that those two strong year-classes still dominate the population about 25 years after they settled. This, thus, is a low bound to longevity. The latter was higher than we had expected for a warm-temperate, shallow-water venerid clam. Other species in the same subfamily (Pitarinae), however, are also reportedly long-lived: 76 years in *Callista brevisiphonata* (Zolotarev 1980) and 40 years in *Callista chione* (Powell and Cummins 1985).

The exploitation of a population that (i) is isolated at the boundary of the species' geographic range of distribution and (ii) depends on very sporadic pulses of recruitment brings up concerns regarding management and conservation. The population may persist because significant longevity bridges long periods with no recruitment ("storage effect"; Warner and Chesson 1985), but reduced survival due to harvesting could shorten the effective length of those bridges. At the same time, however, there is the possibility that settlement of new cohorts was inhibited in a high-density scenario driven by two exceptional year-classes. If that were the case, thinning of the aging population through harvesting could alleviate overcompensatory density dependence, "creating room" for new settlers. These hypotheses could be explored through ex-

TABLE 3.

Comparisons between cohorts and sites (S_1 and S_2) using the likelihood ratio test. H_0 is the null hypothesis; numbers correspond to probability values. Values less than 0.05 are in *italics*. SFD: size-frequency distribution; AGR: size-at-age data from annual growth rings.

Comparison	Site/ Cohort	Estimation Method	H_0 $H_{\infty 1} = H_{\infty 2}$	H_0 $k_1 = k_2$	H_0 $to_1 = to_2$	H_0 $\Theta_1 = \Theta_2$	H_0 $H_{\infty 1}, k_1 = H_{\infty 2}, k_2$
Cohort 1979– Cohort 1980	El Molino	AGR	0.438	0.985	0.499	<i>0.0003</i>	<i>0.035</i>
		SFD	0.315	0.141	0.579	<i>0.00004</i>	0.132
	S_1	AGR	0.795	0.769	0.173	0.167	0.161
		SFD	0.462	0.162	0.656	<i>0.0013</i>	0.112
S_1 – S_2	S_2	AGR	0.676	0.977	0.716	<i>0.0301</i>	0.287
		SFD	0.848	0.516	0.765	<i>0.0024</i>	0.208
	1979	AGR	<i>0.026</i>	0.491	0.246	<i>0.0000004</i>	<i>0.00004</i>
		SFD	0.070	0.524	0.765	<i>0.021</i>	<i>0.014</i>
	1980	AGR	0.078	0.291	<i>0.034</i>	<i>0.00001</i>	<i>0.016</i>
		SFD	0.414	0.688	0.742	0.254	0.136

perimental management, opening sectors of the grounds to the fishery, leaving others as controls, and monitoring the dynamics of recruitment in both treatments.

ACKNOWLEDGMENTS

The authors thank Néstor Dieu, Maite Narvarte, and Sandro Acosta for help during the development of the study and Dr. Ana

Parma for discussion of estimation problems. Cecilia Vinci made available old samples and data (presumed lost) that proved extremely valuable. Funding was provided by fisheries authority of Río Negro Province and by the National University of the Comahue (Argentina). While conducting this study, LO was supported by CONICET (Argentina) and the Pew Foundation Fellows Program in Marine Conservation.

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SEASONAL CHANGES IN BIOCHEMICAL COMPOSITION OF THE CLAM, *EURHOMALEA EXALBIDA* (BIVALVIA: VENERIDAE), FROM THE BEAGLE CHANNEL, ARGENTINA

BETINA J. LOMOVASKY,^{1,2,*} GABRIELA MALANGA,¹ AND JORGE CALVO¹

¹Centro Austral de Investigaciones Científicas (CADIC-CONICET), Ushuaia, Tierra del Fuego, Argentina; ²Ecología, Departamento Biología, FCEyN, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina

ABSTRACT Seasonal changes in biochemical composition (protein, glycogen, and lipid content) of different organs (FV, foot-visceral mass; M, adductor muscles; and GMS, gills-mantle-siphons) of the clam *Eurhomalea exalbida* from Ushuaia Bay (54°50'S, Beagle Channel, Tierra del Fuego) were examined to describe changes in relation to reproduction and growth. A maximum level of protein content was observed during winter in all tissues with other increase during the spawning event (November) in FV and in November and January in GMS. In the adductor muscle, the protein content shows low variability during the year, except in June. These results suggest that all these tissues are involved in the storage of proteins, which are thought to play a role in the gamete maturation. The percentage of glycogen content for FV, M, and GMS showed low values (0.03% to 4.7% AFDW) along the year, which suggests that this component did not have an important function as energetic reserve. Low values of lipid content for FV were found in November with increased levels in summer season. Thus, variations in lipid content can principally be related to gamete emission in November and gamete maturation in summer season. The high levels of lipid content in FV over the rest of the year, except in June, were correlated with the presence of mature gonads throughout the major part of the year. Therefore, our results suggest that in *E. exalbida*, (a) proteins are the most important component stored, and (b) the relative content of protein, lipid, and glycogen of the adult specimens vary seasonally in accordance with the reproductive cycle and the season of maximum shell growth; when the organism reaches reproductive maturity (summer), shell growth slows down because of reproductive investment.

KEY WORDS: Beagle Channel, biochemical composition, bivalve, clams, *Eurhomalea exalbida*

INTRODUCTION

Seasonal changes in biochemical composition (protein, glycogen, and lipid content) may be of great importance in relation to energy metabolism necessary to growth and reproduction (Jayabal & Kalyani 1986, Lodeiros et al. 2001, Navarro et al. 1989). In the tropical scallop *Lyropecten (Nodipecten) nodosus*, it was observed that when the organism reaches reproductive maturity, growth slows down as a result of the reproductive investment, and the biochemical composition may change according to the reproductive requirements (Lodeiros et al. 2001).

The relationship of the energy transfer between different tissues, their capacity of reserve amounts under food availability, and their positive relationship with the high temperature and gonadal maturation have been shown in different species of bivalve mollusks such as scallops (MacDonald & Thompson 1986, Robinson et al. 1981, Sundet & Vahl 1981, Villalaz 1994), mussels (Zandee et al. 1980), and clams (Robert et al. 1993, Urrutia et al. 2001). The scallops *Argopecten ventricosus* (Villalaz 1994), *Chlamys islandica* (Sundet & Vahl 1981), and *Placopecten magellanicus* (Robinson et al. 1981) stored glycogen and lipids in their adductor muscles and digestive gland, respectively, and used them up in gonadal maturation. The mussel *Mytilus edulis* (Zandee et al. 1980) stored glycogen in mantle and digestive gland during the period of food availability to be used in the gametogenic period. Alternatively, some bivalves (i.e., *Abra alba*, *Meretrix meretrix*) can obtain energy directly from food (Ansell 1974a, Jayabal & Kalyani 1986, Lucas 1996).

Biochemical component (lipids, proteins, or carbohydrates) fluctuations have been observed in bivalves and related to the reproductive cycle showing which components were the most important source of energy (Martínez 1991). Bivalves generally store

carbohydrates in large amounts during their growing season and use them over the rest of the year (Beukema 1997); although proteins may be an energy reserve in some bivalve species (Beninger & Lucas 1984, Brockington 2001, Galap et al. 1997). Lipid variation has principally been related to gamete development (Martínez 1991) with the highest levels of lipids during the period when gonads are ripe.

Eurhomalea exalbida (Dillwyn 1817) is a subtidal species with a wide geographic distribution all along the southern tip of South America, from the Chiloe Island (42°S) on the Pacific side (Dell 1964, Osorio et al. 1979, Soot-Ryen 1959) to the Beagle Channel (54°50'S), up to the Buenos Aires province (36°S) on the Atlantic side (Carcelles 1944, Carcelles 1950). *E. exalbida* is commercially exploited throughout the Pacific Coast of South America (Osorio et al. 1979). No records of commercial exploitation exist from the Atlantic side. The Beagle Channel population represents the extreme south of the species' distribution, withstanding large temperature variations (4–11°C), with marked seasonal variation in the biomass of phytoplankton (Hernando, pers. comm.). In the Ushuaia Bay (54°50'S, Beagle Channel), this clam shows a seasonal shell growth pattern (Lomovasky et al. 2002), with adult specimens growing in spring with shell growth slowing down in summer and juveniles growing in both seasons. The parameters of the von Bertalanffy growth function were estimated to be $height_{\infty} = 74$ mm, $k = 0.18$ y⁻¹, $t_0 = 0.15$ y, with a maximum age of 70 y reached (Lomovasky et al. 2002). This species was characterized by an important spawning event in November, followed by a quick recovering in summer with the presence of ripe gonads in the rest of the year (Morriconi et al. 2002). The monthly variations of the energetic content of different organ groups show a positive relationship between higher energy content and the presence of ripe gametes (Lomovasky et al. 2001).

Seasonal variations in growth and reproduction have been correlated with changes in the energy content of different organs (Jobling 1994, Lucas 1996), thereby reflecting the spatial and tem-

*Corresponding author. E-mail: lomovask@mdp.edu.ar

poral energy distribution within the organism. This distribution closely reflects the seasonality of the cost of reproduction and growth and the capacity to accumulate reserves within the organism (Martínez & Mettifofo 1998, Navarro & Torrijos 1995, Robinson et al. 1981, Zandee et al. 1980). Thus, the aim of the current work was to describe the biochemical seasonal changes in different tissues of the clam *E. exalbida* from Ushuaia Bay and the relationship between the observed variations with the gametogenic activity and the maximum shell growth season.

MATERIALS AND METHODS

Samples of *E. exalbida* were collected in Ushuaia Bay (54°50'S, Beagle Channel; Lomovasky et al. 2002) in a subtidal flat at a depth between 2 and 4 m at low tide. Seasonal sampling was conducted by scuba diving between June 1999 and March 2000. Twenty to 30 clams, of shell length ≥ 38 mm (size at first maturity; Morriconi et al. 2002), were sampled each time ($n = 120$) and kept in aquarium for 24 h. Shell length (anterior-posterior axis, SL), measured to the nearest 0.01 mm and total weight (TW, ± 0.1 g) were recorded for each individual. Monthly mean surface temperature was recorded. The minimum mean seawater temperature (4.6°C) was recorded in August and the maximum (8.5°C) was recorded in February (Fig. 1).

After removing the valves, the sex of each individual was determined using gonadal smears. The soft parts were separated into three groups: foot-visceral mass (gonad, digestive gland, and gastrointestinal tract; FV), adductor muscles (M), and gills-mantle-siphons (GMS). These divisions were made based on the presence or absence of gonads in the tissues and on their potential capacity to fulfill a storage function (Lomovasky et al. 2001). The soft parts were dried at 80°C to constant weight and stored at -20°C until processing for biochemical analysis. Ash content was determined by igniting a subsample of tissue in a muffle furnace at 450°C for 24 h.

Condition Index

The relationships between shell length and FV dry mass, M dry mass, and GMS dry mass are represented (Lomovasky et al. 2002) by:

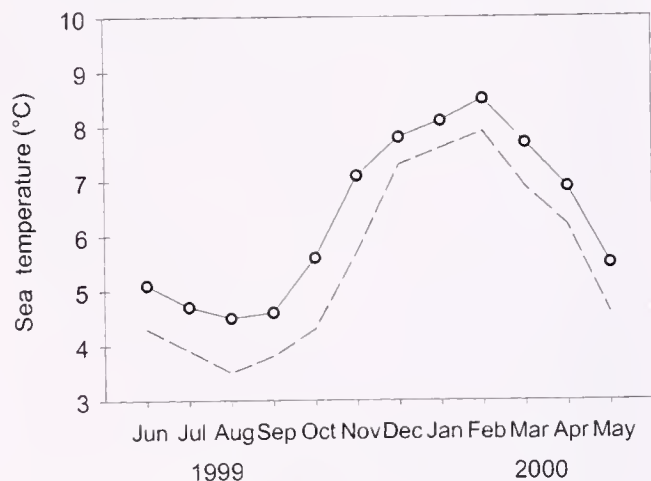


Figure 1. Monthly means of seawater temperature in Ushuaia Bay with maximum and minimum values between June 1999 and May 2000.

$$\log(\text{mass}) = a + b \times [\log(\text{SL})] \quad (1)$$

Seasonal condition values were analyzed using the condition index:

$$\text{CI} = \text{mass/shell length}^b \quad (2)$$

where b is the slope in (1), and were normalized using the relative condition index:

$$\text{RCI} = (\text{CI} - \text{mean CI}) / (\text{SD of CI}) \quad (3)$$

To evaluate the null hypothesis of no difference between seasons in relative condition index in each of the organ groups (FV, M, and GMS), we performed an analysis of variance (ANOVA). The assumptions of normality and homogeneity of variances were tested, and the appropriate transformations were applied when necessary. Unplanned comparisons (modified Tukey test) were made when significant differences were found (Sokal & Rohlf 1995, Zar 1984).

Biochemical Composition

The biochemical composition of dry tissue subsamples from the three organ groups of approximately 20–30 individuals per season was analyzed. Proteins were determined after hydrolysis with NaOH 0.5 N and using BSA 1 mg/mL as standard following the method of Lowry et al. (1951). Glycogen of tissues was extracted by boiling with 30% (p/v) KOH and was determined as total carbohydrates using the method of the Anthrone reagent (Carroll et al. 1956, Seifter et al. 1949). Glycogen was quantified after precipitation with 99% (v/v) ethanol and using glucose standard solution containing 20 $\mu\text{g/mL}$. The difference between protein and glycogen content was considered as lipid content. The results were expressed as milligram (mg) biochemical component per gram (g) of tissue ash-free dry weight (AFDW).

Linear regressions were used to determine the possible relationship between the biochemical content (mg protein, glycogen, and lipid AFDW) of the three organ groups (FV, M, and GMS) and the size of the clams (SL) for each sex. As biochemical content (protein, glycogen, and lipid) per gram were found not to be related to the size of the organisms analyzed in this study, we performed an ANOVA to evaluate the null hypothesis of no difference between seasons in the biochemical content in each of the organ groups. The assumptions of normality and homogeneity of variances were tested, and the appropriate transformations were applied when necessary. A nonparametric test was used (Kruskal-Wallis) when required. Unplanned comparisons (modified Tukey test to parametric analysis or multiples contrast test to nonparametric analysis) were made when significant differences were found (Sokal & Rohlf 1995, Zar 1984).

RESULTS

Condition Index

Significant exponential size-mass relationships between FV, M, and GMS shell-free dry mass (SFDW) as dependent variables and shell length (SL) as the independent variable were found in females and males (Table 1).

The analysis of the relative condition index (Fig. 2A) for FV over time showed the highest values in January 2000 in both sexes whereas the lowest values occurred in June, September, and November for females and in September, November, and March for males (modified Tukey test, $P < 0.05$).

TABLE 1.

Size-mass relationships in *Eurhomalea exalbida* from Ushuaia Bay, Beagle Channel, as described by linear regression $\log Y$ (SFDM) = $a + b \times \log X$.

Y	X	Females				Males			
		a	b	r ²	n	a	b	r ²	n
FV	SL	-5.832	3.3199	0.64	109	-5.615	3.2104	0.45	97
M	SL	-5.1593	2.6909	0.63	109	-5.1156	2.661	0.48	97
GMS	SL	-4.9685	2.7252	0.73	109	-4.9496	2.7223	0.61	97

SL, shell length; FV, foot-visceral mass; M, adductor muscles; GMS, gills-mantle-siphons; SFDM, shell-free dry mass. All significant relationships ($P < 0.05$).

No significant changes in the relative condition index over time were found for either M or GMS in either sex (one-way ANOVA, $P > 0.05$, Figs. 2B and 2C).

Biochemical Composition

No significant dependence ($P > 0.05$) was observed for the relationships between the protein, glycogen, and lipid content and the size of the clams (SL) for each sex. Biochemical content (protein, glycogen, and lipid) per gram were found not to be related to the size of the organisms analyzed in this study.

Foot-Visceral Mass

The mean protein content (Fig. 3A) was significantly different between sexes (two-way ANOVA, $F_{\alpha=0.05,1,110} = 31.22$; $P < 0.001$). Significant higher values in June and November 1999 with minimum values in September 1999 and January 2000 (two-way ANOVA, $F_{\alpha=0.05,4,110} = 72.79$, $P < 0.001$; modified Tukey test, $P < 0.05$) in both sexes were observed. No significant differences were found in the protein content of FV attributable to date-sex interaction ($F_{\alpha=0.05,4,110} = 2.37$, $P > 0.05$).

Glycogen content was comparatively lower than the other components and varied between 0.2% to 4.6% in both sexes (Fig. 3B). No significant differences were found in the glycogen content between sexes (one-way ANOVA, $F_{\alpha=0.05,1,118} = 0.11$, $P > 0.05$). There were significant differences in glycogen content over time (Kruskal-Wallis; $H = 48.77$, $P < 0.001$) with the lowest values in June and November and the maximum value in January (multiples contrast test, $P < 0.05$).

Significant higher values in the FV lipid content in September 1999 and January 2000 in both sexes (for females: Kruskal-Wallis, $H = 37.31$, $P < 0.001$; for males: Kruskal-Wallis, $H = 39.60$, $P < 0.001$) were observed (Fig. 3C).

Adductor Muscles

Proteins were the major biochemical component of the adductor muscle (Fig. 4A). The average protein content showed significant differences between sexes (one-way ANOVA, $F_{\alpha=0.05,1,118} = 5.29$, $P < 0.05$), with slightly higher values in June 1999 in both sexes (females: Kruskal-Wallis, $H = 10.39$, $P = 0.034$; males: one-way ANOVA, $F_{\alpha=0.05,4,54} = 33.80$, $P < 0.001$; modified Tukey test, $P < 0.05$).

The mean glycogen content of M over time for females ($n = 61$) and males ($n = 59$) were significantly different (one-way ANOVA, $F_{\alpha=0.05,1,118} = 4.26$, $P < 0.05$). The differences among

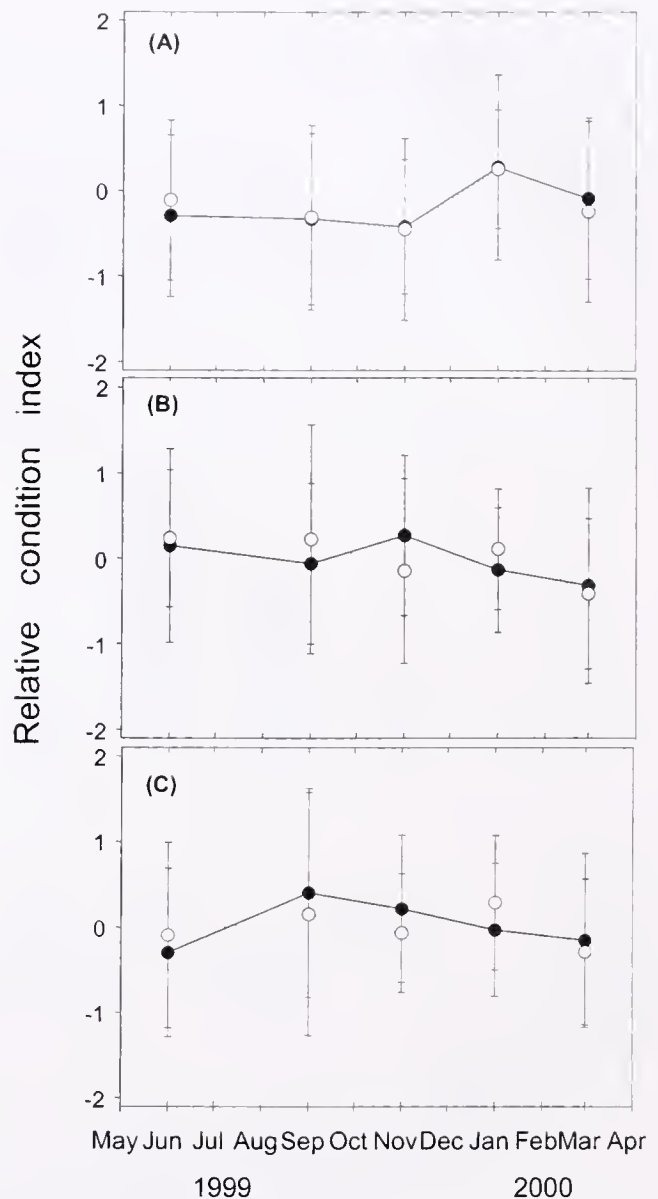


Figure 2. Relative condition index over time in *E. exalbida* from Ushuaia Bay: (A) foot-visceral mass, (B) adductor muscle, (C) gills-mantle-siphons. (●) females, (○) males.

the months were highly significant in both females (one-way ANOVA, $F_{\alpha=0.05,4,56} = 11.67$, $P < 0.001$) and males (Kruskal-Wallis, $H = 32.32$, $P < 0.001$). It was shown in the unplanned comparisons (modified Tukey test) that the glycogen content for females was significantly lower in June and September 1999 ($P < 0.05$) and higher in January and March 2000. Higher significant values in glycogen content among males occurred in November 1999 and January 2000 ($P < 0.05$) than in the rest of the year (Fig. 4B).

Significant differences in the lipid content (Fig. 4C) were found between sexes (Kruskal-Wallis, $H = 5.35$, $P < 0.05$), and over time for females (Kruskal-Wallis, $H = 11.27$, $P < 0.05$) and males (Kruskal-Wallis, $H = 33.49$, $P < 0.001$). Lipid content varied significantly among males during June 1999 and was significantly less ($P < 0.05$) than in the rest of the year. For females, the

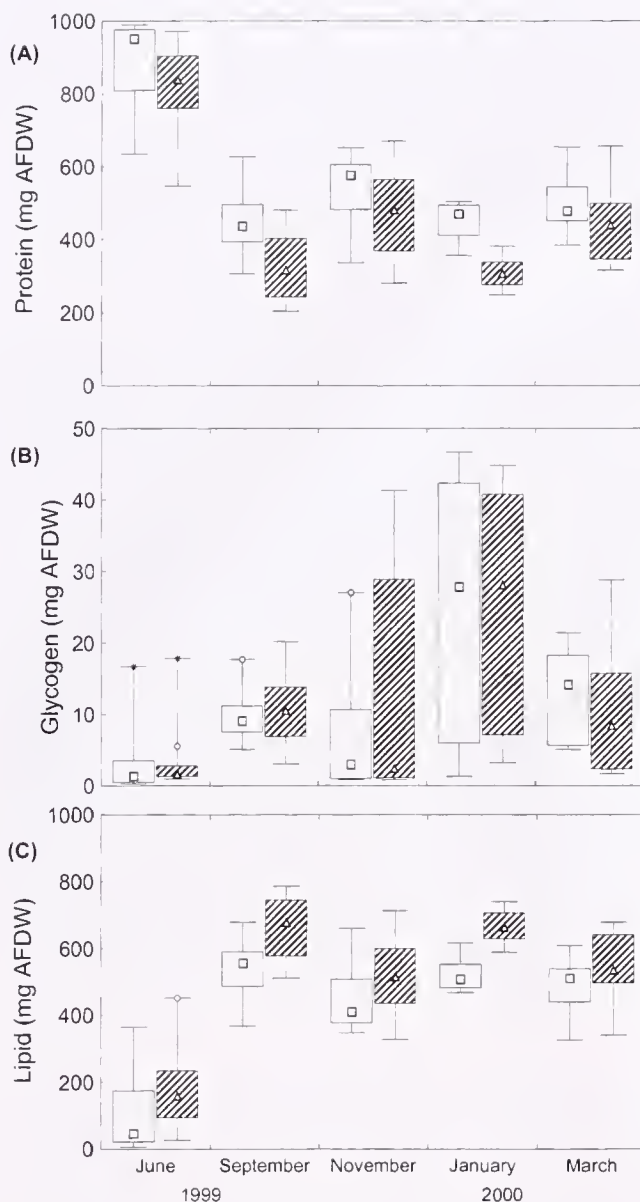


Figure 3. Seasonal variation in the biochemical components for foot-visceral mass (FV) in *E. exalbida* from Ushuaia Bay: (A) protein, (B) glycogen, and (C) lipid. Here and thereafter box plots are constructed with limits of boxes being the 75th and 25th percentile, lines represent maximum and minimum values, points inside boxes are medians, circles are outliers. White boxes: females; hatched boxes: males.

difference found was between the lesser value in June 1999 and the higher value in November 1999 ($P < 0.05$).

Gills-Mantle-Siphons

Protein content (Fig. 5A) was significantly the highest of the year in June and November 1999 and January 2000 in females (Kruskal-Wallis, $H = 42.59$, $P < 0.001$) and males (one-way ANOVA, $F_{\alpha=0.05,4,54} = 9.37$, $P < 0.001$). The average protein content was significantly different between sexes (one-way ANOVA, $F_{\alpha=0.05,1,118} = 12.53$, $P < 0.001$).

No differences (one-way ANOVA, $F_{\alpha=0.05,1,118} = 0.11$, $P > 0.05$) were observed between the mean values in glycogen

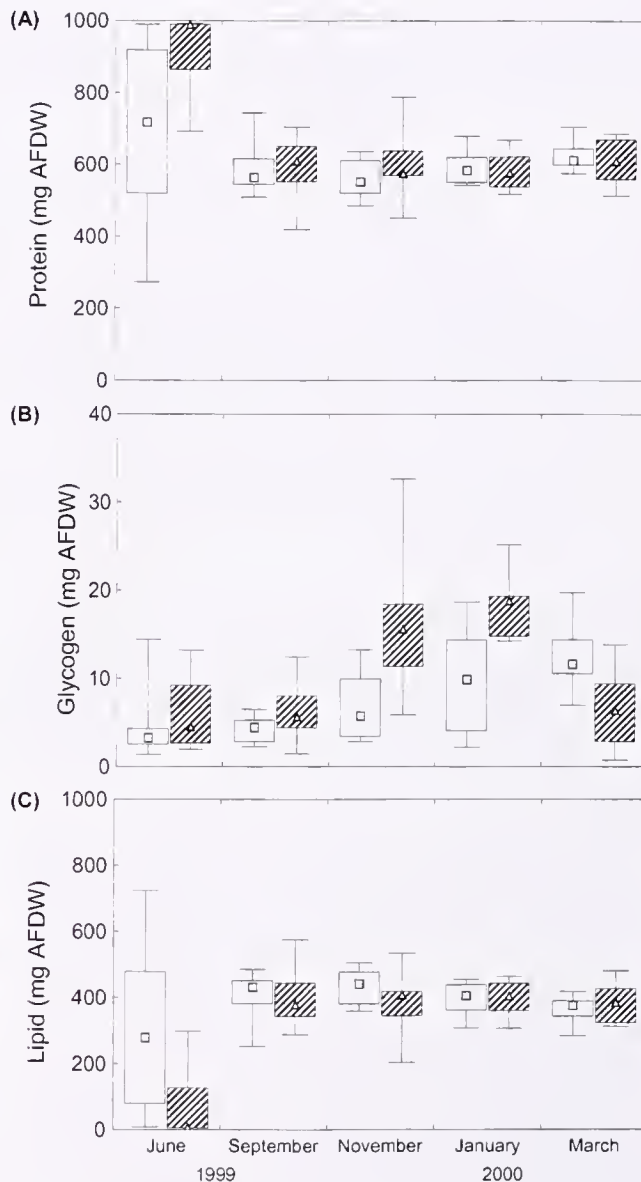


Figure 4. Seasonal variation in the biochemical components for adductor muscle (M) in *E. exalbida* from Ushuaia Bay: (A) protein, (B) glycogen, and (C) lipid. White boxes: females; hatched boxes: males.

content of females and males (Fig. 5B). There were significant differences in glycogen content over time (Kruskal-Wallis, $H = 44.73$, $P < 0.001$) with the lowest values in June 1999 and the maximum value in January 2000 (multiples contrast test: $P < 0.05$).

Significant differences in lipid content between sexes (Kruskal-Wallis, $H = 11.13$, $P < 0.001$) and over time in females (Kruskal-Wallis, $H = 42.55$, $P < 0.001$) and males (Kruskal-Wallis, $H = 26.21$, $P < 0.001$) were observed (Fig. 5C). Lipids were significantly highest in September 1999 and March 2000 in both sexes (multiples contrast test, $P < 0.05$).

DISCUSSION

The relative content of protein, lipid, and glycogen of the three organ groups for the adult specimens of *E. exalbida* from the

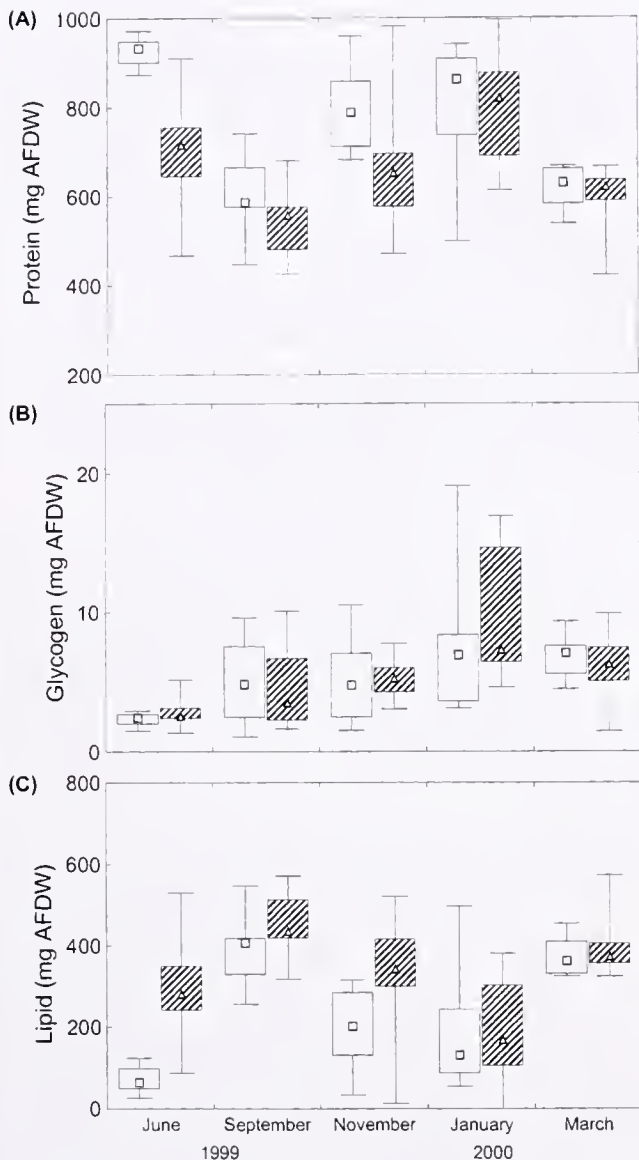


Figure 5. Seasonal variation in the biochemical components for gills-mantle-siphons (GMS) in *E. exalbida* from Ushuaia Bay: (A) protein, (B) glycogen, and (C) lipid. White boxes: females; hatched boxes: males.

Beagle Channel vary seasonally. These changes are principally related to the reproductive cycle and the season of maximum shell growth. Similar characteristics have been observed in other bivalves such as *Anomalocardia squamosa* (Morton 1978), *Donax trunculus* (Ramón et al. 1995), *Euvola (Pecten) ziczac* (Lodeiros & Himmelman 2000), *Lyropecten (Nodipecten) nodosus* (Lodeiros et al. 2001), *Macoma balthica* (Ankar 1980), *Mercenaria mercenaria* (Peterson & Fegley 1986), *Placopecten magellanicus* (MacDonald & Thompson 1986) and *Venus verrucosa* (Arneri et al. 1998).

The seasonal variations of the relative condition index for FV of *E. exalbida* were correlated with the gametogenic cycle (Morriconi et al. 2002) and the monthly relative condition index (Lomovasky et al. 2001) previously observed for this species. The lower RCI value was found in November in coincidence with a higher percentage of partially spawned stage (58.33% for males

and low mean number of mature oocytes for female; Morriconi et al. 2002), and the higher RCI value in January was coincident with the gamete maturation. For M and GMS, no significant changes in mass were observed among the year.

The seasonal biochemical analysis of the FV group showed low values of lipid content in November with an increased level in summer season. We suggest that the energetic variations in FV observed previously (kJ/g AFDW, Lomovasky et al. 2001) could principally be related to the lipid content variations produced by gamete emission in November and gamete maturation in summer season (Morriconi et al. 2002). Similar characteristics were found in *Glycymeris glycymeris* from South Brittany, France (Galap et al. 1997), *Macoma balthica* (Beukema & De Bruin 1977), and *Mytilus edulis* from Dutch Wadden Sea (Pieters et al. 1980, Zandee et al. 1980). The reproductive cycle of *E. exalbida* (Morriconi et al. 2002) showed the presence of mature gonads (60–100%) throughout the major part of the year in both sexes, corresponding with the high levels of lipid content in FV over the year, which has been observed in this study.

Glycogen is the major carbohydrate stored in bivalves (Beukema 1997). However, small amounts of free sugars are always present as well (Whyte & Englar 1982). The glycogen content for FV, M, and GMS found in *E. exalbida* was comparatively lower than the other components (0.03% to 4.7% AFDW), in contrast with other studies in bivalves where high values occurred (31.9% to 48.4%; Giese 1966, Paez-Osuna et al. 1993). Nevertheless, scarce glycogen stored in another burrowing bivalve, *Ensis siliqua*, has been observed (Martínez et al. 1997).

Under conditions of food scarcity, it has been suggested that glycogen from muscular tissues acts as the primary energy reservoir for the formation of gametes in bivalves, such as *Glycymeris glycymeris* (Galap et al. 1997). A reduction of this reserve in storage organs is also commonly correlated with an increase in gonadal lipids (Fernandez-Reiriz et al. 1996, Mann 1979). In the adductor muscle of *E. exalbida*, glycogen content increased during the spawning event (November, in males) and the gamete maturation (summer, males and females) and decreased in the rest of the year, suggesting that glycogen is used in these months but in a very low proportion (<3.3%). Thus, these changes had not been detected in a direct calorimetric analysis (Lomovasky et al. 2001). Therefore, the adductor muscle in *E. exalbida* does not have glycogen storage functions for energy reserves in contrast with previous reports for several bivalves such as scallops (Ansell 1974b, Martínez 1991, Robinson et al. 1981, Sundet & Vahl 1981, Villalaz 1994), mussels (Bayne et al. 1983, Lowe et al. 1982), and oysters (Fernandez Castro & Vido de Mattio 1987).

In FV, the glycogen content was higher in September and January, and a similar tendency for lipid content was observed. The positive correlation between the proportions of carbohydrates and lipids seems to be the rule rather than the exceptions (Beukema 1997). As described above for M, the glycogen content for FV and GMS showed the lowest values along the year, suggesting that this component did not have an important function as substrate for energy reserves in this species.

Alternatively, proteins may be an energy reserve in adult bivalves, particularly during gametogenesis (Beninger & Lucas 1984, Galap et al. 1997). In *E. exalbida*, a higher level of protein content was observed in winter in all tissues, with other increase during the spawning event (November) in FV and in November and January in GMS. In the adductor muscle, with the exception of June, the protein content was approximately constant during the

rest of the year. These results suggest that all these tissues could be involved in the storage of proteins in winter and which are thought to play a role in the gamete maturation. A higher level of protein content in winter has also been observed in digestive gland and gonad of *Spondylus leucacanthus* from the Gulf of California (Rodríguez-Astudillo et al. 2002). It seems therefore that in *E. exalbida*, proteins may be the more important component stored.

The energetically convenient degradation of glycogen has been studied in several species, such as *Mytilus edulis* (De Zwaan & Eertman 1996), *Scapharca inaequivalvis*, and *Venus gallina* (Brooks et al. 1991), related to the type of metabolic pathway used during environmental anaerobiosis when the metabolic rate is strongly suppressed compared with the aerobic resting rate (De Zwaan & Eertman 1996). *E. exalbida* is a subtidal, Sub-Antarctic species whose cold-waters habitat provides it with oxygen. Emergence and anoxia-adapted physiology (De Zwaan & Eertman 1996) may not be related to these species. The protein seems to be its only alternative resource of energy under conditions of food scarcity. However, it cannot be certain without further studies and proper investigation about the possible advantage of using protein as an energy reserve and the mechanisms of regulation (e.g., anti-freezing proteins).

A relationship between the greater availability of phytoplankton (Hernando, pers. comm.), sea temperature, and gamete maturation with a slowed down shell growth has been suggested for this species (Lomovasky et al. 2001, Lomovasky et al. 2002, Morriconi

et al. 2002). Thus, food availability may be an important source of the nutrients required for the gonadal ripening process. Seasonal variation in temperature and the availability of food appear to be closely related to the energy available for growth and reproduction in other bivalve species (Beukema & De Bruin 1977, Griffiths & King 1979, Newell & Branch 1980, Jayabal & Kalyani 1986, MacDonald & Thompson 1986, Mann 1979, Navarro et al. 1989, Smaal et al. 1997, Sukhotin 1992, Zandee et al. 1980). In *E. exalbida* from Ushuaia Bay, shell growth in spring (Lomovasky et al. 2002) occurred during spawning (Morriconi et al. 2002). In summer, the shell growth slowed down, and gamete maturation was found whereby energy was allocated to reproduction rather than to growth. In contrast, juvenile organisms grew up such as in spring and in summer (Lomovasky et al. 2002). The differences detected between juvenile and adult organisms could be related to the energy partition between growth and reproduction.

ACKNOWLEDGMENTS

The authors thank Daniel Aureliano and Adalberto Ferlito for technical assistance, Dr. Oscar Iribarne, Dr. Susana Puntarulo, and Lic. Marcelo Hernando for their critical reading of the manuscript, and Laura Perlstein for correcting the English manuscript. This work was partially supported by a grant from Fundación Antorchas (Project 13817/4). B. J. L. and G. M. were supported by a fellowship from CONICET (Argentina).

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SEPARATION OF RECENTLY SETTLED MANILA CLAMS, *TAPES PHILIPPINARUM* (A. ADAMS AND REEVE, 1850), FROM THREE SEDIMENT TYPES USING SUCROSE DENSITY SOLUTION

DAPHNE M. MUNROE,¹* DOUG BRIGHT² AND SCOTT MCKINLEY¹

¹Center for Aquaculture and the Environment, Faculty of Agricultural Sciences, University of British Columbia, West Vancouver, BC, Canada, V7V 1N6; ²UMA Engineering Ltd., Victoria, BC, Canada V8T 2W1

ABSTRACT Thorough study of the life history of important fishery and aquaculture shellfish species is essential for the most appropriate management and development of the resource. The early juvenile life stages of wild intertidal clam populations are difficult to study because of their existence in sediments of similar size and color to the animal. Here, we test the efficacy of methods for separation of recently settled Manila clams (*Tapes philippinarum*) from different types of intertidal sediments using density. Three aquaria were set up with sterilized sediments of each of the three sediment types—cobble/sand/shell, cobble/mud, mud/sand—resulting in nine aquaria total for the experiment. Each tank was filled with filtered, sterilized sea water; the water was heated to 20°C and aerated. Competent *T. philippinarum* larvae were added to each tank at a known density and were allowed to metamorphose and settle. Aquaria were then drained, and four sediment samples were taken from each aquarium for evaluation of number of juvenile clams present. Samples were wet-sieved to isolate the size fraction from 125 to 500 µm, and then this size fraction was settled through high-density (1.9 g/mL) sucrose solution to separate stained clams from sediments. The average numbers of clams counted per sample for each sediment type were 57.9 (SD = 37.1) for mud/sand, 60.0 (SD = 36.1) for cobble/sand/shell, and 53.1 (SD = 39.6) for cobble/mud. The average number of clams expected per sample based on the number of larvae added to each tank was 58.8 for all three sediment types. Statistical analysis revealed no significant difference between the mean number of clams per sample and the expected value of 58.8 for all three sediment types. Therefore, these results indicate that the methods used here can be used to extract rapidly recently settled Manila clams from all sediment types with a high degree of accuracy.

KEY WORDS: density separation, juvenile clams, sampling, sucrose, *Tapes philippinarum*

INTRODUCTION

The Manila clam (*Tapes philippinarum*, A. Adams and Reeve, 1850) is of major importance to both the wild fishery and aquaculture industry worldwide. In British Columbia, Canada, this species is non-native and is thought to have been introduced along with oyster seed from Japan in the 1930s (Bourne 1982). Since its introduction, *T. philippinarum* has become a very important species economically and is the basis of the current clam culture industry (Jones et al. 1993). The conditions in British Columbia are favorable for the Manila clam, and it has become well established throughout the southern coastline (Quayle 1974).

In the general bivalve life cycle, a pelagic larval stage is followed by metamorphosis, during which the swimming organ or velum is lost, and the bivalve transforms into the benthic or epibenthic juvenile form, depending on the species. The postmetamorphic juvenile stage of the Manila clam is found in the upper layers of the sediment; however, sampling juveniles from these sediments presents many problems because of the similar coloration and size of the clams and fine sediments. Consequently, very few studies have focused on this life stage. When sampling has been carried out and early juveniles in the sediment are counted (Glock 1978, Jones 1974, Williams 1980), the work is extremely time-consuming and prone to error. Rarely, however, is reference made in published studies of bivalve settlement and early life-stage ecology to the accuracy of the sorting and counting methods involved. A simple, consistent, and effective method for separation of postsettlement bivalves from the sediments would allow for more studies to be carried out and more insight into the recruitment patterns of the Manila clam and other valuable clam species to be gained.

The density of juvenile postsettlement clams and cockles was estimated to be 1.036–1.076 g/mL in a study by de Montaudouin (1997) and 1.1 g/mL in a study by Jonsson et al. (1991), whereas minerals are more dense, typically with a specific weight of 2.5 g/mL and higher (Denny 1993). Density gradients of silica sols have been used in many studies to separate lighter meiofauna from higher density sediment fractions (Burgess 2001, Nichols 1979, Schwinghamer 1981). It has been shown that high-density sucrose solutions can also be used to separate meiofauna from muddy organic sediment (Heip et al. 1974). The technique explored herein involves wet sieving to isolate the size fraction of the sediment containing the bivalves, then allowing that size fraction to settle through a high-density sucrose solution (1.9 g/mL) to isolate the meiofauna and allow for easier counting of the bivalves. This technique will be tested using three different sediment types to determine the efficacy of the method with various sediments.

The clam juveniles would be expected to float in a solution with a density of 1.9 g/mL. However, the high concentration of sucrose increases the osmotic pressure on the animal cells causing them to dehydrate, thereby increasing the density of the animals and causing them to sink (Bowen et al. 1972, Price et al. 1978). Although the juveniles become more dense and sink, they sink slowly in relation to the higher density mineral components of the sample and are found in the top layer of the sediment once it has settled out. This process of isopycnic sedimentation at one solute density to separate particles of different densities is called "rho spectrometry" by Price et al. (1977).

MATERIALS AND METHODS

Three different sediment types were obtained from intertidal beaches north of Nanaimo, British Columbia. Nine tanks were prepared with each of the three different sediment types (treatments) randomly assigned to three tanks per sediment type. The

*Corresponding author. E-mail: dmmunroe@interchang.ubc.ca

sediment types were cobble/mud, cobble/sand/shell and mud/sand according to their properties, and a subsample (approximately 20 cm³) of each sediment type was analyzed for grain size components by wet sieving using methods adapted from Komar (1998). Organic material was not removed prior to wet sieving. Briefly, the sediment was wet-sieved, dried at 60°C (Boyd & Tucker 1992) for 30 min, and weighed.

Sediments to be used in the experiment were autoclaved prior to placement in each aquarium. Each aquarium was filled to a depth of 5 cm (surface area of each tank was 800 cm²) with the assigned sediment type, and then filled with 15 L seawater (filtered to 1 µm and sterilized with UV radiation). All aquaria were aerated and warmed to 20°C before addition of competent, hatchery-reared Manila clam larvae. A total of 2400 clams were added to each of the nine tanks.

Tanks were maintained at 20°C and clams were fed a combination of 50:50 *Chaetoceros muellerii* and *Isochrysis spp* (Tahitian strain) at a combined concentration of 20,000–30,000 cells/mL (Jones et al. 1993). Clams were left in the tanks for 11 days to settle and metamorphose. The tanks were then drained to simulate low-tide sampling conditions, and core samples of the sediment were taken. Four sediment cores were taken from each of the nine tanks (36 samples total). A small corer made of PVC pipe (5 cm internal diameter) was inserted 1 cm into the sediment, and a thin metal lid was slid under the pipe to prevent the sediment from falling out. Samples were placed in a plastic sample bag, labeled, and frozen for later counting. Freezing was chosen as a method of sample preservation to ensure consistency between laboratory methods and field methods that have been used for a parallel study.

For enumeration, samples were thawed then placed in 0.01% phloxine B dye for at least 20 min (Williams 1978). Samples were then washed through a series of sieves; the fraction of sediment from 125 to 500 µm was placed into a high-density sucrose solution (1.9 g/mL) in 30-mL test tubes. The test tubes were inverted to mix the sediments, to avoid particle-particle interactions (Price et al. 1977), then left to settle out by gravity (minimum of 25 min). The top layer of sediment was pipetted off the surface of the settled sediments in the test tube. The amount of sediment pipetted off the top was approximately 1–2 mL, which is less than 10% of the original core sample volume. The number of clams in each sample was counted under a dissecting microscope at 60× magnification. Statistical analysis of variance was calculated using JMP statistical software.

RESULTS

The grain size for each sediment type is shown below in Table 1. The cobble/mud sediment contained the largest fraction of the

>2000-µm size category with 82% by weight. The cobble/sand/shell also contained mostly >2000-µm-sized components; however, it should be noted that in this case, over half of this size category was composed of broken shell whereas the >2000-µm fraction in the cobble/mud was entirely small cobble. The mud/sand sediment was mostly composed of medium sand (70% dry weight); with coarse sand and fine sand making up another 18% dry weight. The components >2000 µm were only broken shell in the mud/sand sediment.

Two thousand four hundred clams were added to each tank, and each tank had a sediment surface area of 800 cm²; therefore, the expected average density of clams in each tank was 3 clams/cm². The surface area of each core was 19.7 cm², so the expected average number of clams per core was 58.8. The means and 95% confidence limits for number of clams recovered from coring the three sediment types is shown in Figure 1. Comparison of the mean numbers of clams per core among the three sediment types was done to test if all means were equal regardless of sediment type. The mean values for clams per core were 60.0 for cobble/sand/shell, 53.1 for cobble/mud, and 57.9 for mud/sand. Based on analysis of variance, there was no significant difference in the number of juvenile clams recovered from each of the different sediment types and the expected value 58.8 ($n = 3$, probability ranged from 0.63 to 0.94). Nor was there a significant difference between treatments ($n = 3$, probability = 0.90). Individual t -tests were also run to determine if the recovery of clams from coring of any of the experimental units (nine tanks × three treatments) departed significantly from the expected value based on larval seeding density. There was no statistically significant difference between the observed and expected values for any of the experimental units ($n = 4$, probability ranged from 0.30 to 0.75).

DISCUSSION

Analysis of grain size components (Table 1) shows that there were large differences between the compositions of the three sediment types used in this experiment. This was important because we were testing the accuracy of the sampling methods for extraction and counting of postsettlement juveniles in different types of sediments. For example, the mud/sand sediment was composed of 70% medium sand, which means 70% of the entire sample was the same size class in which the bivalves are found. This has the potential to lead to difficulty and inaccuracy in extraction of the clams from this large sediment fraction. In this experiment, no difficulties were encountered: The numbers of clams counted per core did not differ in the three types of sediment. Furthermore, for all three sediment types, the number of clams counted in each sample did

TABLE 1.
Grain size components, percentage by dry weight, of each sediment type.

Size Class	Size Range (µm)	% Overall Weight		
		Cobble/Mud	Cobble/Sand/Shell	Mud/Sand
Shell	(>2000)	—	43	4
Granule+	(>2000)	82	30	—
Very coarse sand	(1000–2000)	3	8	3
Coarse sand	(500–1000)	4	7	11
Medium sand	(125–500)	7	9	70
Fine sand	(75–125)	2	2	7
Silt	(<75)	2	1	5

The size category >2000 µm contains both granule+ and broken shell.

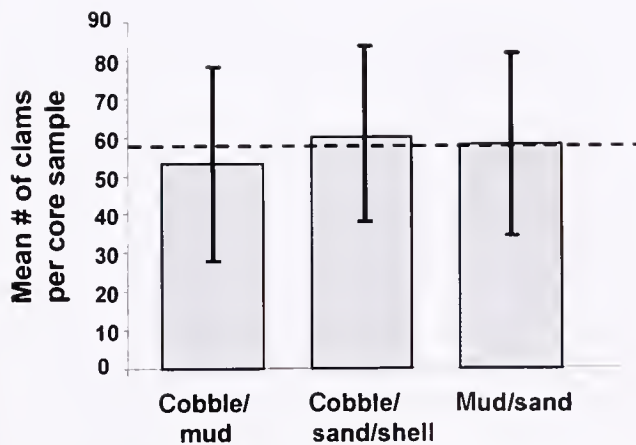


Figure 1. Means and 95% confidence limits for numbers of clams per sample for the three sediment types. The dashed line indicates the expected number of clams per sample (58.8) based on the number of larvae placed in each tank. ($n = 3$ for each treatment).

not differ from the value expected per sample based on the number of clams initially placed in tanks. This means that with this method, there was an estimated 100% recovery of bivalves from sediment regardless of sediment type.

Individual tanks within each sediment type were tested to ensure that mean numbers of clams counted per sample did not differ statistically from one tank to the next. Large standard errors were seen in data from some individual tanks. These were overcome when all samples of each sediment type were analyzed together. This may be interpreted as a result of the patchy settlement of clam larvae (Williams 1980), especially in coarser and heterogeneous substrates, and implies that the sampling effort may need to be increased for such sediment types.

The recovery of 100% of the bivalves that were placed in tanks also suggests 100% survival from the time of introduction of the larvae to the time of recovery of postsettlement clams. Survival rates for *T. philippinarum* in a hatchery generally vary from 50–90% depending on larval quality (Utting & Spencer 1991). The recovery of an estimated 100% of added larvae (had the entire sediment surface been sampled) is probably due to the relatively short duration of the study. Some of the postsettlement clams, in fact, may have been nonviable or dead at the time of sampling, but freezing and subsequent staining would not distinguish recently deceased clams from live ones. In other circumstances, it might be expected that some mortality would occur prior to settlement, so a failure to account for 100% of the introduced larvae might not be attributable to the sorting techniques in other studies.

Separation and counting of live clams prior to freezing was not attempted, leaving some question about whether separation as described herein would be equally effective for live specimens or those preserved using other methods such as formalin fixation. Schwinghamer (1981) conducted tests on live separation of benthos from mud and sediments using centrifugation in sorbitol

and Percoll and found it to be an effective separation method that allowed for proper identification and observation of sampled benthos.

In a study by Burgess (2001), density separation of meiofauna from sediment was carried out using Ludox. Sediment samples were mixed with Ludox, then centrifuged to separate the meiofauna. Using this method, Burgess was able to recover 95.9% of the bivalves in the sample. Jonge and Bouwman (1977) also found the use of density separation of nematodes and copepods from sediment and detritus to be more effective and accurate than hand-sorting decantation methods. Both Burgess (2001) and Jonge and Bouwman (1977) note that a potential shortcoming of the density separation method is that animals may attach to sediments and therefore sink with them. Postmetamorphic bivalve juveniles have the ability to attach to larger sediments using a byssus. In this study, the sediment fractions larger than 500 μm were not examined to look for attached juveniles; however, recovery was estimated at 100% in the size fraction examined, so few if any clams were likely to have been found in larger fractions. It is possible that if there were any clams attached to sediments by byssal threads, the threads were released when the sediment samples were frozen.

The use of the sucrose-density separation method described here is effective for counting newly settled juvenile clams from sediment. The use of the high-density sucrose solution to isolate the lower density animals increases sampling efficiency by decreasing the time to sort through sediment. Decreasing the time to sort increases sampling accuracy because less physical and psychologic variance is introduced (Price et al. 1977). This decrease in sorting time would be especially important for sediments like the mud/sand sediment used here, where sieving would result in retention of the majority of the sediments along with the bivalves and, thus, hand sorting and counting would be quite tedious and prone to error.

These results show that these methods can be used in the field with the confidence to count recently settled clams in sediment samples involving a variety of sediment types. This would be useful in the assessment of farming techniques and questions of industry sustainability and environmental protection. Juvenile density estimates are important in the understanding of settlement patterns and early mobility in this economically important species. More generally, such studies are crucial to the understanding of bivalve larval ecology, demographics, and environmental/climatic factors that control recruitment, as well as invasion rates of exotic bivalves.

ACKNOWLEDGMENTS

We would like to acknowledge the generous donation of lab space and larvae by the Center for Shellfish Research at Malaspina University-College; particularly the help of Dr. Bill Pennell for his guidance and enthusiasm, Gord Edmondson for help with equipment preparation, and Jenny Dawson-Coates for help with algal rearing and feeding. Also, the authors thank Terri Sutherland for her helpful comments.

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ACETYLCHOLINE AND SEROTONIN INDUCE LARVAL METAMORPHOSIS OF THE JAPANESE SHORT-NECK CLAM *RUDITAPES PHILIPPINARUM*

PAULA M. URRUTIA,¹ KEN OKAMOTO² AND NOBUHIRO FUSETANI^{1*}

¹Laboratory of Aquatic Natural Products Chemistry, Department of Aquatic Biosciences, and

²Laboratory of Aquatic Conservation, Department of Ecosystem Studies, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

ABSTRACT The effects of neuroactive compounds on larval metamorphosis of the Japanese short-neck clam *Ruditapes philippinarum* (Heterodonta, Veneridae) were investigated by exposing pediveligers to acetylcholine, serotonin, epinephrine, norepinephrine, dopamine, L-3,4-dihydroxyphenylalanine (L-DOPA), carbamylcholine, and succinylcholine at concentrations of 1, 10, and 100 μ M. Larval metamorphosis with 100 μ M serotonin was 80.7%, and that with 10 μ M and 100 μ M acetylcholine was 92.9% and 70.6%, respectively. No significant differences were observed in the induction activities of epinephrine (35.1–36.1%), L-norepinephrine (2.1–20.3%), L-DOPA (6.4–15.8%), and nontreated groups (0–5.6%). Dopamine showed no inducing activity. Treatment with 100 μ M carbamylcholine induced 37.6% of metamorphosis in 23-day-old larvae. Larval metamorphosis rate increased significantly with exposure time when treated with acetylcholine, carbamylcholine, and serotonin. Low postlarval survivorship after treatments might not be related to toxicity of chemicals but due to an energy deficiency after an accelerated completion of metamorphosis in neurochemically stimulated larvae. The effectiveness of inducer drugs was observed to be age-dependent. Nineteen-day-old larvae reacted less than 23-day-old ones from the same cohort. Larvae of *R. philippinarum* responded differently to neurotransmitters from bivalves of the subclass Pteriomorpha (i.e., Ostreidae, Pectinidae, Mytilidae) which are more sensitive to catecholamines and L-DOPA. This difference suggests that the mechanisms triggering metamorphosis may differ among bivalve groups.

KEY WORDS: acetylcholine, bivalve, larval age, metamorphosis, neuroactive compounds, postlarval survivorship, *Ruditapes philippinarum*, serotonin, short-neck clam

INTRODUCTION

Many marine benthic invertebrates produce planktonic larvae. At the end of the planktonic period, larvae respond to complex environmental and biologic stimuli leading to settlement and metamorphosis, allowing the onset of juvenile and adult benthic life forms. This transition is modulated by chemical cues of various biologic origins (Hadfield & Paul 2001). Such cues bind to specific receptors located in the larval neural tissues, triggering metabolic pathways resulting in the behavioral and morphologic changes typical of the metamorphosis process (Burke 1983, Sarojini et al. 1999). Neurotransmitters, their precursors, or similar molecules have been reported to mimic the natural chemical cues and are known to trigger larval settlement and metamorphosis in a variety of marine invertebrates (Hadfield 1984, Morse 1985, Morse 1990, Pires & Hadfield 1991, Okamoto et al. 1995). In bivalves, catecholamines and L-3,4-dihydroxyphenylalanine (L-DOPA) were described as highly effective inducers in *Mytilus edulis* (Cooper 1982), *Crassostrea virginica* (Coon et al. 1986), *Ostrea edulis* and *C. gigas* (Shpigel et al. 1989), *C. belcheri* (Tan & Wong 1995), *Pecten maximus* (Chevolot et al. 1991), and *M. galloprovincialis* (Satuito et al. 1999). Furthermore, acetylcholine was reported as a settlement inducer for *C. gigas* (Beiras & Widdows 1995) and gamma aminobutyric acid increased the settlement rate of *Pinctada margaritifera* (Doroudi & Southgate 2002). Despite their ecological and economical importance, few studies have assessed larval settlement and metamorphosis of clams: soft bottom burrowers like *Mercenaria mercenaria* (Bachelet et al. 1992, Woodin et al. 1995), *Mulinia lateralis* (Luckenbach 1984, Grassle et al. 1992), *Spisula solidissima* (Snelgrove et al. 1998), and *Mya arenaria* (Snelgrove et al. 1999) have been studied for settlement and habitat selection in terms of hydrodynamics. However, biologic and

chemical regulation of these processes is largely unknown in clams. We investigated the effects of neuroactive compounds on larval metamorphosis of *Ruditapes philippinarum*, focusing on a search for highly effective inducers, the postmetamorphic survivorship, and a larval age dependent response. The Japanese short-neck clam is an important commercial resource that sustains fisheries and aquaculture. Recently, disease outbreaks (Hamaguchi et al. 1998, Allam et al. 2000, Renault et al. 2001) along with seasonal (Calvez & Guillou 1998) and environmental fluctuations (Ishii et al. 2001) have diminished wild and farmed populations of this clam. Effective chemicals for induction of larval metamorphosis may improve aquaculture practices and add to our understanding of factors controlling settlement and metamorphosis in clams.

MATERIALS AND METHODS

Larval Cultures

Groups of the Japanese short-neck clam *R. philippinarum* (total shell length: 27.5 ± 2.3 mm, $n = 40$) were collected during reproductive seasons (May and October 2001) from Hamana Bay, Japan ($34^{\circ}41'33''$ N, $137^{\circ}35'58''$ E). Spawning was induced by combining cycles of exposure to cool air (Satuito et al. 1994), immersion in a sperm solution (Loosanoff & Davis 1963) obtained by stripping of 1–2 mature males, and thermal stimulation (Toba et al. 1994). Finally, the clams were kept individually in 50-mL beakers filled with 1- μ m filtered seawater (FSW) at 20°C for spawning. Released ovules were suspended in FSW in a glass container. Similarly, released sperm was collected and suspended. Adding drops of the sperm solution, a mixture of ovules from several female clams was fertilized. Further on, fertilized zygotes were washed with FSW, placed in 1-L beakers containing fresh FSW, and kept undisturbed for embryonic development at 25°C (Toba 1992). Straight-hinge veligers were collected 20 to 24 h after fertilization, washed with GF/F-FSW (Whatman GF/F glass-fiber filters), and placed in 1-L glass containers filled with GF/F-

*Corresponding author. E-mail: anobu@mail.ecc.u-tokyo.ac.jp

FSW under the following conditions: Initial density of 10 larva/mL (Toba et al. 1994), 25°C constant temperature (Toba 1992), and salinity of 29–32 p.s.u. throughout the culture period. Aeration was provided for the larval culture containers through glass Pasteur pipettes during the entire culture period. Larval age in days was counted from the first day of larval culture (first straight-hinge veliger). Daily *ad libitum* food ration was given as follows: An initial diet of *Pavlova lutheri* at 3×10^5 cells/mL (final concentration) was provided until day 5, after which a mixture of *Chaetoceros calcitrans* and *Pavlova lutheri* was given at 4×10^5 cells/mL until the culture was finished (Toba et al. 1994). Microalgae for the larval diets were cultured in K medium (Keller & Guillard 1985) at 25°C and constant illumination. Early pediveligers were observed from days 12 to 14 of culture. Pediveliger culture densities were kept at 1.5 larva/mL. Two experiments were carried out with actively swimming pediveligers. Larval survivorship of the cultures used for experiments 1 and 2 was 24.1% and 31.3%, respectively.

Experiment 1: Effects of Neuroactive Compounds on Larval Metamorphosis

Six neuroactive compounds, acetylcholine chloride (Wako Pure Chemical Industries, Ltd), serotonin creatinine sulfate (Wako), epinephrine bitartrate (Research Biochemicals Inc.), L-norepinephrine bitartrate (Wako), dopamine hydrochloride (Wako) and L-DOPA (Wako) were tested at three concentrations (1, 10, and 100 μ M) in triplicate against a control group. Stock solutions were prepared by dissolving in FSW at several folds of the final concentration. Filtered seawater was used for the control group and dilution of chemical compounds. Eight hundred forty-seven 22-day-old pediveligers were distributed at a density of approximately 1–2 larva/mL in 57 10-mL glass beakers containing FSW. The corresponding solution of neuroactive compound was added to adjust the final concentrations to 1, 10, and 100 μ M and final volume to 10 mL in each beaker. Only FSW was added to three of the beakers containing larvae to adjust to the final volume of 10 mL; these were used as control groups. Because oxidation of catechols was expected to occur in seawater, larvae were placed into fresh FSW and newly prepared chemical solutions every 12 h. This procedure was applied to all treatments, subjecting the larvae to the same conditions. Onset of metamorphosis was monitored after 24 and 48 h of exposure. The criteria to determine whether a larva had metamorphosed were by visual detection of postlarval shell growth, complete loss of velum, and observation of the gill rudiment activity using a stereomicroscope and an inverted microscope. Metamorphosed larvae are referred to as postlarvae. After 48 h exposure to the chemicals, larvae and postlarvae were washed, placed in neuroactive-compound-free FSW, and maintained for 24 h to monitor post-treatment survivorship and other effects such as late metamorphosis response. Larvae were not fed during the assay. The experiment was run at 22°C in a dark, temperature-controlled room at the Fisheries Laboratory of the University of Tokyo, Hamana Bay, in June 2001.

Experiment 2: Effects of Larval Age and Neuroactive Compounds on Larval Metamorphosis

Based on the results obtained in experiment 1, this assay was conducted to evaluate exclusively the cholinergic agonists acetylcholine chloride, carbamylcholine chloride (Nacalai Tesque Inc.), and succinylcholine chloride (Wako), and serotonin creatinine sulfate on the metamorphosis of a cohort of larvae of three age

groups, 19, 21, and 23 days old. This assay was run in triplicate against a control group. The concentrations of chemicals were adjusted to 1, 10, and 100 μ M and an exposure time of 48 h. A sample of pediveligers was taken from the culture and subjected to the induction assay; remaining siblings were kept in culture until the indicated ages at a density of approximately 1.5 larva/mL and with aeration to avoid settlement. Five hundred fifty 19-day-old larvae and 437 and 339 21- and 23-day-old larvae, respectively, were distributed in 10-mL beakers in a similar way as in experiment 1. Larvae were not fed during the assay. Metamorphosis was monitored after 1, 5, 12, 24, 36, and 48 h of exposure. The trials were run in triplicate against triplicate control groups of FSW under the same conditions as in experiment 1.

Data Analysis

The Kolmogorov-Smirnoff test ($\alpha = 0.05$) was used for evaluation of normality. Metamorphosis percentages were analyzed using Kruskal-Wallis analysis of variance (ANOVA) on ranks followed by Dunnett multicomparison ($\alpha = 0.05$). The effect of each chemical on larval metamorphosis was classified as positive when the metamorphosis percentage was higher than the control group and as negative when the metamorphosis percentage was equal to or lower than that of the control group. The metamorphosis response at 24 and 48 h in each treatment was compared using a *t*-test ($\alpha = 0.05$). A Kruskal-Wallis ANOVA on ranks followed by a Dunnett multicomparison test ($\alpha = 0.05$) was used to analyze the postlarval survivorship among compounds and concentrations. A two-way ANOVA followed by a Tukey test ($\alpha = 0.05$) for pairwise comparison was used to assess the effect of larval age on the response to three concentrations of serotonin and choline compounds with the data arcsine square root transformed.

RESULTS

Effects of Neuroactive Compounds on Larval Metamorphosis

Larval metamorphosis of *R. philippinarum* induced by neuroactive compounds varied with concentration. Positive responses were observed for five of the six chemicals tested (Fig. 1). Acetylcholine at 10 μ M showed the highest percentage metamorphosis after 48 h (mean \pm SD $n = 3$, $92.9 \pm 12.4\%$) followed by serotonin at 100 μ M ($80.7 \pm 18.0\%$) and acetylcholine at 100 μ M ($70.6 \pm 14.2\%$). Metamorphosis percentages obtained with these treatments were significantly higher than that of the control group ($P < 0.05$). Larvae exposed to either 1 μ M acetylcholine or 1 μ M serotonin did not metamorphose during the 48-h assay period. Positive chemicals showed no differences in induction activities at 24 h and 48 h of exposure ($P > 0.05$), except for acetylcholine at 10 and 100 μ M ($P < 0.001$ and $P = 0.005$, respectively), where the metamorphosis was induced within 24 h. The metamorphosis percentage was significantly increased when larvae were exposed to higher concentrations of acetylcholine ($P = 0.021$) and serotonin ($P = 0.028$). Although epinephrine showed a positive effect, there were no statistical differences between epinephrine and the control group ($P = 0.166$). Larvae of *R. philippinarum* metamorphosed in response to L-norepinephrine and L-DOPA, yet they were less effective, yielding low percentage metamorphosis, and were not significantly different from the control group. Dopamine showed no inductive effect at all concentrations tested after 48 h of

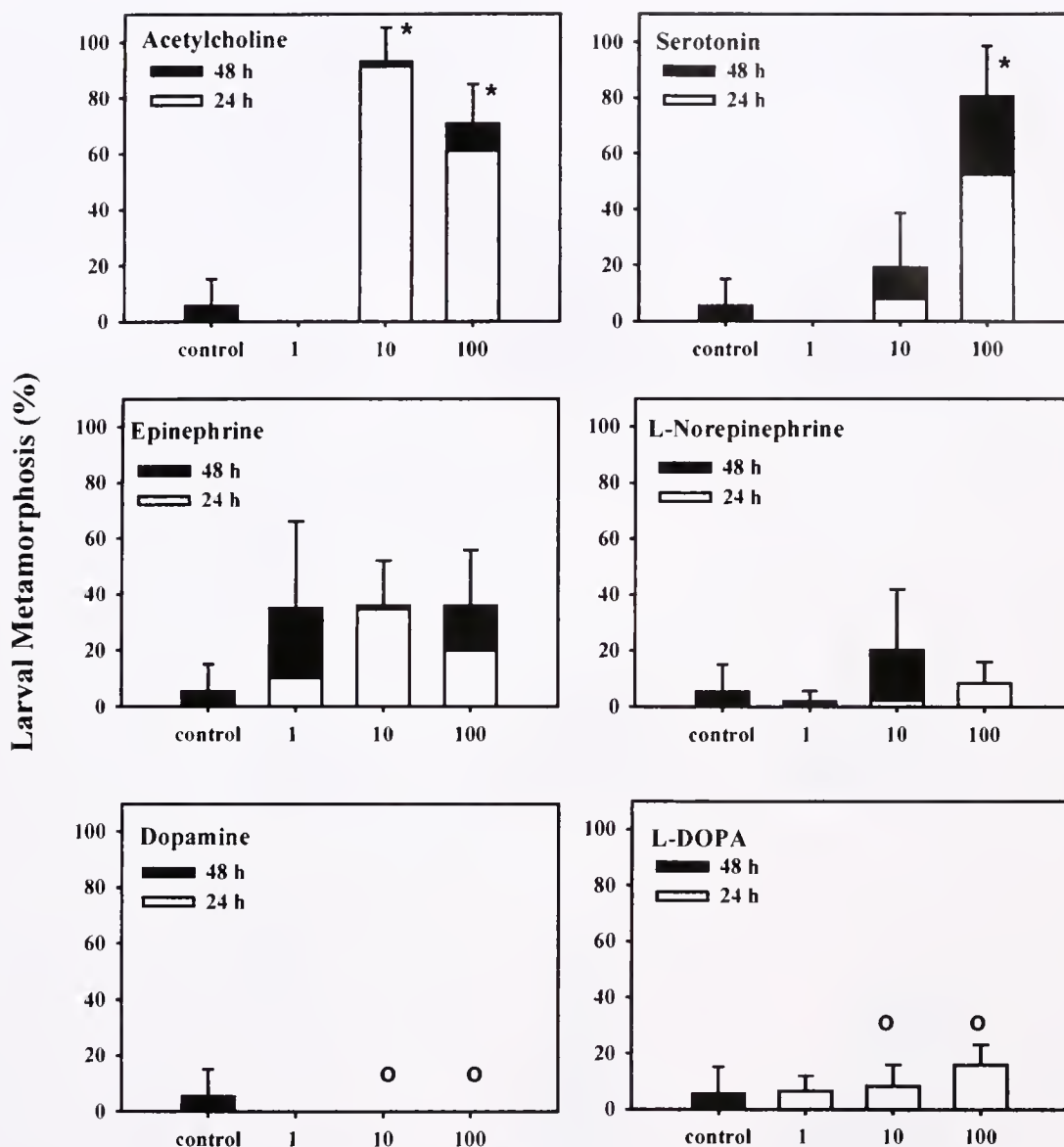


Figure 1. Metamorphosis of *Ruditapes philippinarum* induced by neuroactive compounds. Bars indicated cumulative mean metamorphosis percentage after 24 and 48 h ($\pm\text{SD}$, $n = 3$). Asterisk (*) indicates differences to the control (FSW). Circle (○) indicates 100% larval and postlarval mortalities after 48 h of exposure.

exposure. Larval mortalities were observed when exposed to dopamine and L-DOPA at concentrations of 100 and 10 μM .

Larvae exposed to the effective chemicals changed their regular swimming pattern, combining crawling and short swimming near the bottom. A progressive extension of the crawling period was observed to be dependent on exposure time. Vela in the pediveligers lost most of their cilia. When there was no movement, we assumed they were metamorphosing, because larvae and postlarvae always crawled before and after metamorphosis; no byssal attachment was observed.

Effects of Neuroactive Compounds on Postlarval Survivorship

After assays, larvae and postlarvae were transferred to fresh FSW to observe post-treatment responses after 24 h. Postlarval survivorships are summarized in Table 1. Variability was observed in the postlarval survival rate after exposure to neurochemicals for 48 h. All postlarvae died after exposure to L-DOPA in all concen-

trations tested. Larvae that did not undergo metamorphosis died in all cases studied, except for the control group and the 1 μM acetylcholine, 1 μM serotonin, and 1 μM dopamine treatments, where remaining pediveligers displayed active swimming or crawling behavior. In these trials, newly metamorphosed larvae were observed (acetylcholine, $4.3 \pm 3.8\%$; serotonin, $14.2 \pm 1.6\%$; and dopamine, $7.8 \pm 6.9\%$) and recorded as post-treatment metamorphosis (Table 1). No significant differences ($P = 0.944$) in postlarval survivorships at the concentrations tested were observed among treatments. This result indicates that concentrations of 1, 10, and 100 μM of the neurochemicals used for metamorphosis induction may not have affected the postlarval survivorship, except for treatments with L-DOPA at the higher concentrations, which led to 100% postlarval mortalities. The percentage survivorship of postlarvae after the treatments with acetylcholine, serotonin, epinephrine, and L-norepinephrine was not significantly different from that of the control group (100% survival) ($P = 0.062$).

TABLE 1.

Postlarval survivorship of *Ruditapes philippinarum* 24 h after treatment with neurocompounds.

Compounds	1 μ M	10 μ M	100 μ M
Acetylcholine	4.3 \pm 3.8*	5.2 \pm 4.5	30.1 \pm 6.9
Serotonin	14.2 \pm 1.6*	92.2 \pm 7.2	22.0 \pm 11.1
Epinephrine	23.7 \pm 26.7	13.7 \pm 5.2	6.5 \pm 6.7
L-norepinephrine	5.4 \pm 5.1	19.2 \pm 5.4	0
Dopamine	7.8 \pm 6.9*	0	0
L-DOPA	0	0	0

Pediveligers were exposed to neuroactive-compounds solutions for 48 h. Immediately after the induction assay, postlarvae were transferred to FSW for 24 h to observe survivorship. Figures indicate mean survival percentage \pm SE ($n = 3$). Postlarvae of the control group (FSW) exhibited 100% survival.

* Completion of metamorphosis observed 24 h after the chemical treatment (post-treatment metamorphosis).

Effects of Larval Age and Neuroactive Compounds on Larval Metamorphosis

Ruditapes philippinarum pediveligers of the three age groups (19, 21, and 23 days old) from the same cohort showed a distinct metamorphosis rate when exposed to various concentrations of choline derivatives and serotonin (Fig. 2). The highest metamorphosis percentages were obtained when 21-day-old larvae were exposed to 10 μ M serotonin and 23-day-old larvae to 10 and 100 μ M serotonin (mean \pm SD $n = 3$, 71.4 \pm 49.5%, 61.8 \pm 18.6%, and 64.0 \pm 10.3%, respectively), of which only the responses of 23-day-old larvae to 10 and 100 μ M serotonin were significantly higher than the control group ($P < 0.05$). Lower metamorphosis percentages yet significantly different from control groups were observed in 21-day-old larvae treated with 1 and 100 μ M acetylcholine and 100 μ M carbamylcholine ($P < 0.05$). Percentage metamorphosis of 23-day-old larvae treated with acetylcholine 100 μ M was also significant ($P < 0.05$). Among age groups, metamorphosis percentages obtained when exposed to FSW (control group) were not significantly different ($P = 0.089$). The larval age was found to influence significantly larval metamorphosis induced by acetylcholine ($P < 0.001$), carbamylcholine ($P < 0.001$), and serotonin ($P = 0.011$). Younger larvae exhibited the lower response to the chemicals. A significant increase in the percentage metamorphosis was observed when 19-day-old larvae were compared with 23-day-old larvae ($P < 0.05$). Responses of 21- and 23-day-old larvae to acetylcholine, carbamylcholine, and serotonin were not significantly different ($P > 0.05$). Metamorphosis rate did not increase with exposure time when larvae of the 19-day-old group were stimulated with neuroactive compounds ($P = 0.059$, Fig. 3). A significant time-dependent response was observed for larvae of 21-day-old ($P = 0.003$) and 23-day-old ($P < 0.001$) when treated with acetylcholine, carbamylcholine, and serotonin. It should be noted that the onset of metamorphosis was observed after 5-h exposure for 23-day-old larvae, which subsequently metamorphosed within 24 h. Percentage metamorphosis did not significantly increase for concentrations of serotonin ($P = 0.331$), acetylcholine ($P = 0.123$), and carbamylcholine ($P = 0.175$). Acetylcholine and carbamylcholine were effective at lower and higher concentrations. Similar trends were observed for acetylcholine in experiment 1. Serotonin was more effective at higher concentra-

tions for older larvae. Effectiveness of serotonin at the highest concentration tested was observed in experiment 1 as well. However, the percentage metamorphosis of 21-day-old larvae when exposed to 100 μ M serotonin was similar to that of the control group ($P = 0.269$). This outcome was in contrast to the effectiveness of 100 μ M serotonin observed in experiment 1 and later with older sibling larvae. Succinylcholine exhibited the least effect; no significant differences among age groups and doses were observed (19-day-old, $P = 0.392$; 21-day-old, $P = 0.392$; 23-day-old, $P = 0.278$).

DISCUSSION

Effects of Neuroactive Compounds on Larval Metamorphosis

Our investigation demonstrated that the neuroactive compounds serotonin and acetylcholine induced larval metamorphosis of the Japanese short-neck clam *R. philippinarum*. We first tried to identify highly effective inducer neuroactive compounds in terms of high postlarval yield. We assessed different types of neuroactive compounds: the choline derivative acetylcholine, the indolamine serotonin, and the catechols epinephrine, L-norepinephrine, dopamine, and L-DOPA. Among the compounds tested, acetylcholine (10 and 100 μ M) and serotonin (100 μ M) exhibited the highest inducing activity on larval metamorphosis. Soon after exposure to these chemicals, pediveligers exhibited a behavioral shift from swimming to crawling actively on the bottom. Most larvae completed metamorphosis in 48 h. In contrast, only weak activity of serotonin at the same concentration ranges was reported for *Mytilus galloprovincialis* (Satuito et al. 1999), *Crassostrea gigas* (Beiras & Widdows 1995), and *Patinopecten yessoensis* (Kingzett et al. 1990). Acetylcholine was previously reported to be ineffective for *M. galloprovincialis* (Satuito et al. 1999), but an "active" inducer for *C. gigas*, though only 20–40% of larvae metamorphosed (Beiras & Widdows 1995). Recently, it was reported that acetylcholine induced <40% metamorphosis in *Mytilus edulis* larvae (Dobretsov & Qian 2003). Interestingly, the catecholamines, epinephrine and norepinephrine, and L-DOPA were not effective inducers of larval metamorphosis of *R. philippinarum*. The ineffectiveness of epinephrine and norepinephrine was also observed for the Venus clam *Ruditapes largillierii* (Kent et al. 1999). Thus, clam species of the genus *Ruditapes* (subclass Heterodonta) seem to exhibit a different response to neuroactive compounds than bivalves in the subclass Pterimorphia (i.e., Ostreidae, Pectinidae, Mytilidae), which have routinely been induced to metamorphose by epinephrine, norepinephrine, or L-DOPA. Our results suggest that acetylcholine and serotonin may play roles in the metamorphosis of *R. philippinarum*. Regardless of the internal regulatory mechanism, solutions of these neuroactive compounds accelerated metamorphogenic changes in *R. philippinarum* pediveligers. In the absence of an appropriate chemical inducer, metamorphosis did occur, but at a slower rate, as was observed in the pediveligers incubated in FSW.

Although there are differences in chemicals capable of inducing larval metamorphosis in *R. philippinarum*, behavioral and morphologic changes associated with the end of larval life were rather similar to bivalves reported in other studies. *R. philippinarum* exhibited a change of the typical pediveliger swimming behavior; the larva showed alternating cycles of near-bottom swimming and crawling followed by only crawling on the substrate until the onset of metamorphosis, where no motion was observed until the completion of the final anatomic changes of metamorphosis. How-

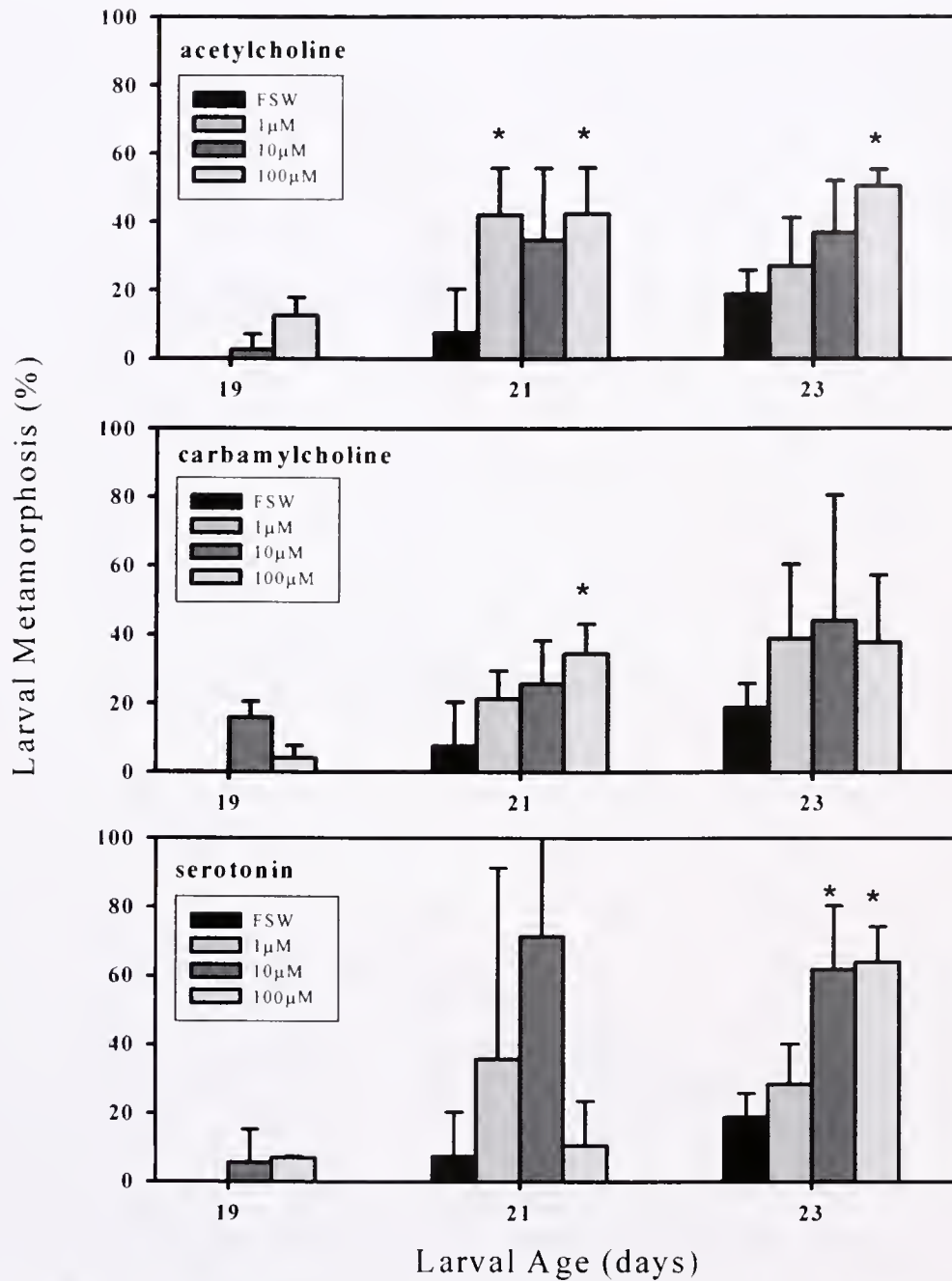


Figure 2. Metamorphosis percentages at three larval ages of a cohort of *Ruditapes philippinarum* when exposed to acetylcholine, carbamylcholine, and serotonin for 48 h. Bars indicate mean \pm SD ($n = 3$). Asterisk (*) indicates significant differences to the control groups (FSW) ($P < 0.05$). Metamorphosis increased significantly with the larval age ($P < 0.05$). 19-day-old larvae were compared to 21- and 23-day-old larvae. Metamorphosis of 21- and 23-day old larvae were similar ($P > 0.05$).

ever, *R. philippinarum* pediveligers do not attach to the substrate prior to metamorphosis; they do not secrete a cementing or fixing substance unlike oysters (Cranfield 1973), mussels (Eckroat & Steele 1993), and scallops (Benninger & Le Pennec 1991, Tapia et al. 1993).

Effects of Neuroactive Compounds on Postlarval Survivorship

Postlarvae of *R. philippinarum* obtained by treatment with neuroactive compounds had a very variable and low survival rate after

treatments. We observed that the survival rate did not differ significantly among all concentrations, but it did differ among compounds. Dopamine and L-DOPA at higher concentrations appeared to be toxic after 48 h of exposure. When treated with acetylcholine, serotonin, epinephrine, and L-norepinephrine, the postlarval survival rate was low. The control group exhibited a remarkable survivorship of 100%. In contrast, with treatments of acetylcholine and serotonin, a much higher percentage of metamorphosed larvae was observed within 48 h of exposure, yet surprisingly, a high

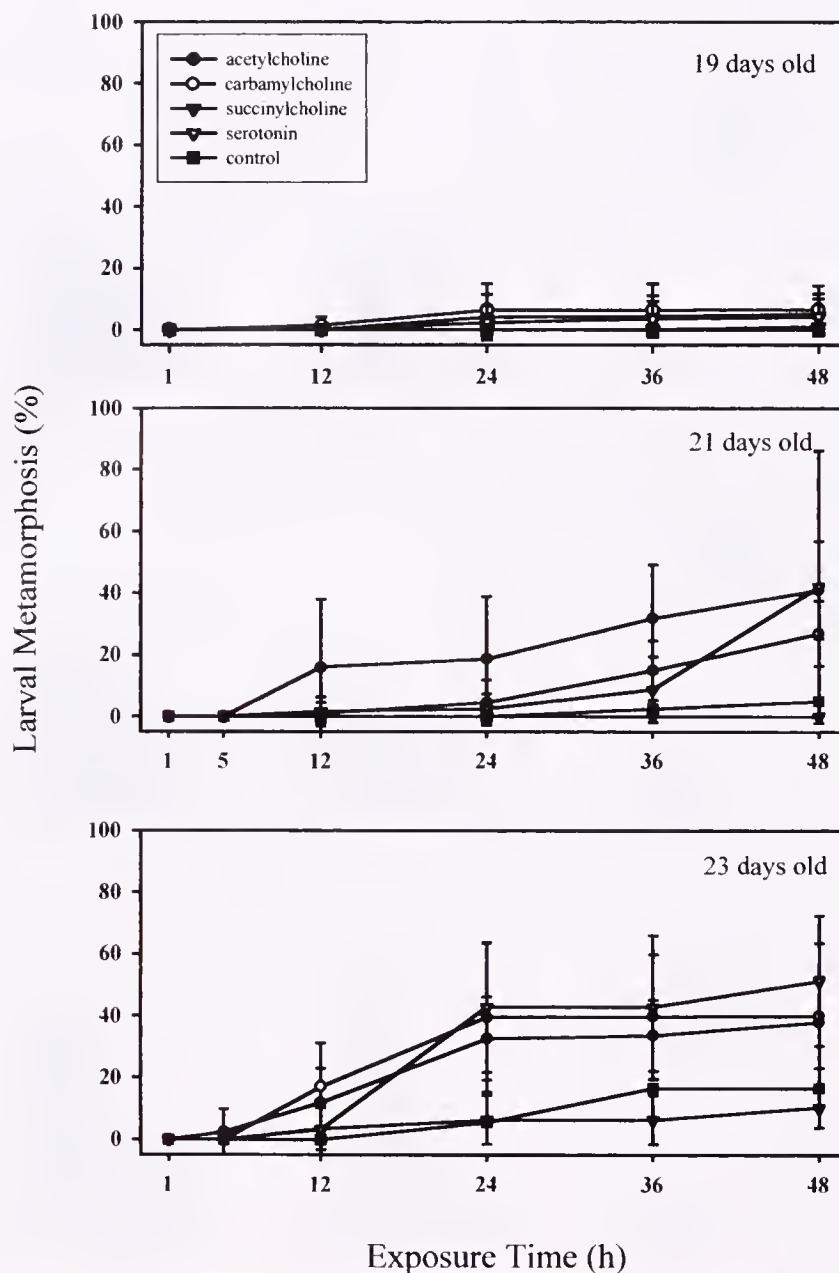


Figure 3. Time-dependent response of a cohort of *Ruditapes phillipinarum* larvae to neuroactive compounds. Plots indicate cumulative mean percentage metamorphosis (\pm SD, $n = 9$).

proportion of those postlarvae was not able to survive. Primarily, this low survival rate seems to be related to the chemical treatment (i.e., a toxicity degree). However, taking into account that our assay was carried out under no feeding conditions—exposing the individuals to over 72 h of starvation—the low postmetamorphosis survivorship may be due to an energy deficiency rather than toxicity of neuroactive compounds. Treatments of effective inducers such as acetylcholine and serotonin may have intervened the neural pathways needed to trigger metamorphosis, accelerating the process (Hadfield 2000). Consequently, more larvae underwent metamorphosis prior than they could replete metabolic reserves to endure metamorphosis. This may explain our low postlarval survival rate under nonfeeding conditions. However, the fact that 100% survival of non-fed “spontaneously” metamorphosed post-

larvae in the control groups impedes reconciling of our results. For this reason, our arguments need to be addressed in future studies. Mortality rates after induction of metamorphosis by chemicals have also been reported for oysters. Haws et al. (1993), in the oysters *C. gigas* and *C. virginica*, found an increment of mortality due to a depletion of lipids, carbohydrates, and proteins 36 h after larval metamorphosis induced by epinephrine. Interestingly, Shpigel et al. (1989) reported no negative effects of epinephrine on the survival rate of *O. edulis* cultchless spats fed daily with a mixed microalgae diet. Consequently, food availability after metamorphosis may be one of the main factors determining postlarval survivorship. Therefore, effective neuroactive compounds may serve as postlarval yield enhancers only when the food supply after the treatment is sufficient.

Effects of Larval Age and Neuroactive Compounds on Larval Metamorphosis

We demonstrated that larval age had a significant effect on larval response to effective neuroactive compounds. The effectiveness of acetylcholine and serotonin was low in 19-day-old larvae treated for 48 h. Sibling larvae only 2 days older exhibited a significantly enhanced response to the same chemicals. Among the older larvae assayed in our experiments (21 and 23 days old), the responses to the effective neuroactive compounds were similar. Thus, it seems that effective neuroactive compounds such as serotonin and acetylcholine have the same effect on metamorphosis of older larvae. Taken together, in the results of both experiments we find support to conclude that *R. philippinarum* pediveligers older than 21 days become competent to metamorphosis in response to acetylcholine and serotonin. The same pattern was observed when larvae were exposed to carbamylcholine, an acetylcholine receptor agonist. This compound exhibited a similar activity-increment to that observed with acetylcholine in the same range of concentrations, though it was less effective. These findings along with those obtained in experiment 1 may suggest that

acetylcholinergic and serotonergic neural pathways play roles in triggering metamorphosis in *R. philippinarum*. The observed age-dependency of the clam's responsiveness to chemical-inducing stimuli may suggest the development of a sensory system at the end of the pediveliger stage for the recognition of specific cues for settlement and metamorphosis (Hirata & Hadfield 1986, Pawlik 1992, Hadfield et al. 2000). We assumed that the effective neuroactive compounds stimulated those receptors directly involved in metamorphosis regulation of the short-neck clam. A great deal of variability in the larval responses to neuroactive compounds was evident, which may be explained in part by the variability in the degree of development among individuals in the cohort. The effectiveness of serotonin and acetylcholine at various larval ages suggests that these compounds could be used to synchronize several larval cohorts for postlarval yield. These may have useful applications in aquaculture production as well as in metamorphosis studies of the Japanese short-neck clams.

ACKNOWLEDGMENTS

The authors thank the anonymous reviewers for their valuable comments on the manuscript.

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PERKINSUS SP. INFECTION RISK FOR MANILA CLAMS, *VENERUPIS PHILIPPINARUM* (A. ADAMS AND REEVE, 1850) ON THE PACIFIC COAST OF NORTH AND CENTRAL AMERICA

RALPH A. ELSTON,¹* CHRISTOPHER F. DUNGAN,² THEODORE R. MEYERS³ AND KIMBERLY S. REECE⁴

¹AquaTechnics, PO Box 687, Carlsborg, Washington 98324; ²Maryland Department of Natural Resources, Cooperative Oxford Laboratory, 904 S. Morris Street, Oxford, Maryland 21654; ³Alaska Department of Fish and Game, PO Box 25526, Juneau, Alaska 99802; ⁴Virginia Institute of Marine Science, PO Box 1346, College of William and Mary, Gloucester Point, Virginia 23062

ABSTRACT Manila clams (*Venerupis philippinarum*, A. Adams and Reeve 1850) are an important aquaculture species on the west coast of North America and are also cultured in Europe, Asia, and other locations. Clams cultured on the west coast of North America are free of *Perkinsus* sp. infections, while clams from certain Asian and European sources are infected. Infection in Korean Manila clams is reportedly associated with high morbidity and mortality. We evaluated the health status of readily accessible Manila clam juveniles from Korea that were proposed for importation into Mexican waters where they would increase in size, and then be shipped into the United States, either to market destinations or to receiving waters. The examination of the clams was performed as a preliminary assessment for a producer considering the importation of Korean Manila clams. We report finding a high prevalence of a *Perkinsus* sp. causing significant tissue damage in juvenile Korean Manila clams. Parasite taxonomic verification was made using a genus-*Perkinsus* SSUrRNA gene-specific DNA probe for *in situ* hybridization. The use of this probe is validated and reported for the first time. As a result of this finding, no importation of this clam stock took place. It is urgently important to make widely known the risk of the spread of this disease into the clam stocks of the west coast of North and Central America to prevent such an introduction. In addition, we report new information regarding the prevalence and intensity of this disease in juvenile clams available for export, as well as pathologic features of the disease.

KEY WORDS: *Venerupis (Tapes) philippinarum*, juvenile clam infection, *Perkinsus* sp., DNA probe, *in situ* hybridization

INTRODUCTION

Manila clams (*Venerupis philippinarum*, A. Adams and Reeve 1850) are an important aquaculture species on the west coast of North America. More than 7 million pounds of littleneck clams, predominantly *V. philippinarum*, were produced in Washington, California, and Oregon in 2000 (Pacific Coast Shellfish Growers Association 2003), and additional production occurs in British Columbia, Canada. Although Alaska produces native littleneck clams, *Protothaca staminea* (Conrad 1837), Manila clams are exotic, and importation for aquaculture purposes is prohibited. *Venerupis philippinarum* is also an important aquaculture species in Europe and Asia, and is infected with *Perkinsus* sp. on both continents. Specifically, *Perkinsus atlanticus* occurs in Europe (Navas et al. 1992), a *P. atlanticus*-like parasite occurs in Japan (Hamaguchi et al. 1998), and *Perkinsus* sp. occurs in Korea (Choi & Park 1997) and China (Liang et al. 2001). Consistent with the close homology noted between DNA sequences at several *P. atlanticus* and *Perkinsus olseni* loci by diverse investigators, Murrell et al. (2002) assert these parasitic species to be synonymous, with taxonomic priority to the *P. olseni* name.

In contrast, clams from the west coast of North America are free of *Perkinsus* sp. infections. A survey of Manila clam health and conditions on the west coast of North America (Pacific Shellfish Institute 2001), and the required examination of over 3000 clams for health certifications from 1991 to 2002, showed no evidence of *Perkinsus* sp. infection. Moreover, such infections have not been reported elsewhere on the west coast during routine annual examinations and frequent health examinations of brood stocks and seed clams since 1985. In addition, *Perkinsus* sp. in-

fection has not been reported in the native littleneck clam *P. staminea* or any other bivalve species from the west coasts of North or Central America.

Manila clams may be imported as a live market product from Korea, Japan, or other Asian countries into North America. In 1998, we evaluated the health status of juvenile Manila clams from Korea that had been proposed for importation into Mexican waters, where they would gain size before shipment to the United States, either to market destinations or to receiving waters for further grow out. The examination of clams was performed as a preliminary assessment for a producer considering the importation of Korean Manila clams. We report the finding of a high prevalence of a *Perkinsus* sp. causing significant tissue damage in juvenile Korean Manila clams.

As a result of this finding, no importation of this clam stock took place. It is urgently important to make widely known the risk of the spread of this disease to west coast North American clam stocks to prevent the introduction of this debilitating and lethal clam parasite. In addition, we report here new information regarding the prevalence and intensity of this disease in juvenile clams that are available for export, as well as pathologic features of the disease. Finally, a novel genus-*Perkinsus* DNA probe for *in situ* hybridization (ISH) assays on histologic samples is described.

Taxonomic references to the Manila clam (also commonly referred to as the Japanese littleneck clam) in the scientific literature are particularly confusing. We have designated the species as *V. philippinarum* in accordance with the Committee on Scientific and Vernacular Names of Molluscs within the Council of Systematic Malacologists, American Malacological Union (American Fisheries Society 1998). The common name Manila clam is also found in the literature, apparently in reference to the same species, associated with scientific designations of *Tapes philippinarum*, *Ruditapes philippinarum*, *Tapes semidecussatus*, and *Tapes japonica*.

*Corresponding author. E-mail: aquatech@olympen.com

TABLE 1.
ISH assay results with genus-*Perkinsus* SSUrRNA probe, Perksp700DIG.

Parasite	Host	Sample	± Probe Hybridization	Sample Source	Reference
<i>Perkinsus</i> sp.	<i>V. philippinarum</i>	98-SH14-5	+	R. A. Elston	this article
<i>Perkinsus</i> sp.	<i>V. philippinarum</i>	98051504-2	+	Y. Maeno	Maeno et al. 1999
<i>P. atlanticus</i>	<i>R. decussatus</i>	685a	+	C. Azevedo	Azevedo 1989
<i>P. olseni</i>	<i>H. laevisgata</i>	ST389-35	+	C. L. Goggio	Goggio et al. 1989
<i>P. chesapeakei</i>	<i>M. arenaria</i>	CHBRMa-14	+	C. Dungan	Dungan et al. 2002
<i>P. andrewsi</i>	<i>M. balthica</i>	MB3a2	+	F. G. Kern	Coss et al. 2001
<i>P. marinus</i>	<i>C. virginica</i>	221, 556-15	+	K. S. Reece	Mackio et al. 1950
<i>P. mediterraneus</i>	<i>O. edulis</i>	08 and 016	+	A. Villalba	Casas et al. in press
<i>Perkinsus</i> sp.	<i>C. pacificus</i>	CH02882	+	C. L. Goggin	Goggin et al. 1989
<i>P. qugwadi</i>	<i>P. yessoensis</i>	6492-A5	–	S. M. Bower	Blackbourne et al. 1998
<i>Haplosporidium nelsoni</i>	<i>C. virginica</i>	201, 239	–	E. Burreson	Haskin et al. 1966
<i>H. costale</i>	<i>C. virginica</i>	196, 774	–	E. Burreson	Couch 1967
haplosporidian-like sp.	<i>P. platyceros</i>	90-568J	–	S. M. Bower	Bower & Meyer 2002
<i>Hematodinium</i> sp.	<i>C. sapidus</i>	98-513	–	J. D. Shields	Shields 1994
<i>Hematodinium</i> sp.	<i>N. norvegicus</i>	990427Nnor-1	–	G. Stentiford	Field & Appleton 1995

MATERIALS AND METHODS

A total of 64 Manila clams [16–32 mm shell length (SL)] from Incheon Bay, South Korea, were clinically examined in February 1998 and were fixed whole in Davidson's shellfish fixative (Shaw & Battle 1957). These tissues were processed for routine histologic examination.

A representative tissue section containing parasites was evaluated by ISH. The genus-*Perkinsus* DNA probe was designed to specifically target SSU rRNA sequences of *Perkinsus* species by aligning the available SSU rRNA gene sequences, while not hybridizing to the sequences of closely related parasite taxa including dinoflagellates and apicomplexans. An SSU rRNA gene sequence is not available for *Perkinsus qugwadi*. The resulting probe Perksp700DIG (5'-CGCACAGTTAAGTRCGTGRGCACG-3') was 5' end-labeled with digoxigenin (Sigma-Genosys, The Woodlands, TX). ISH assays were performed as previously described (Stokes & Burreson 1995, Stokes & Burreson 2001), except that 125 µg/mL pronase was used for permeabilization, instead of proteinase K, for a 30-min digestion, and a probe concentration of 7 ng/µl was used for hybridization. The probe was tested on an array of *Perkinsus* sp.-infected, paraffin-embedded tissues (Table 1), including *Perkinsus marinus* in *Crassostrea virginica*, *P. atlanti-*

cus in *Ruditapes decussatus*, *P. olseni* in *Haliois laevisgata*, *Perkinsus andrewsi* in *Macoma balthica*, *Perkinsus* sp. in *Vereurupis philippinarum* from Japan, *Perkinsus chesapeakei* in *Mya arenaria*, *Perkinsus mediterraneus* n. sp. in *Ostrea edulis* (Casas et al. in press), *Perkinsus* sp. in *Chama pacificus*, and *P. qugwadi* in *Patinopecten yessoensis*. Probe specificity was validated by testing tissue sections of the blue crab *Callinectes sapidus*, which was infected with the parasitic dinoflagellate *Hematodinium* sp. (Shields 1994), *Hematodinium* sp.-infected Norway lobster *Nephrops norvegicus* (Field & Appleton 1995), *Haplosporidium nelsoni*-infected and *Haplosporidium costale*-infected *C. virginica* oysters, and spot prawn *Pandalus platyceros*, infected by an undescribed haplosporidian-like protozoan parasite (Bower & Meyer 2002). Replicate sections of nonspecific ISH assay signal controls of each sample were tested identically, except that they received hybridization buffer without probe during the overnight hybridization step.

RESULTS

Histologic Evaluation of Infected Clams

The prevalence of juvenile clams infected with the presumptive *Perkinsus* sp., was 59 of 64 (92%), based on histologic examination. The protozoa were systemically distributed in a variety of organs, most typically in subepithelial areas of the gills, and fre-

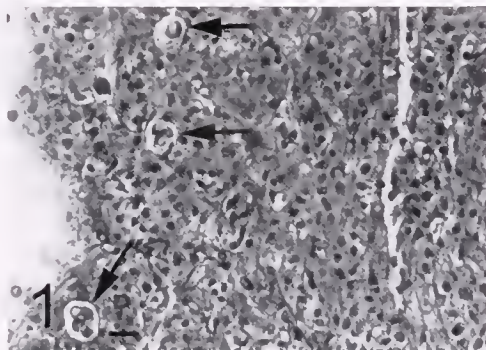


Figure 1. Gill tissue of a juvenile Korean Manila clam infected with *Perkinsus* sp. (arrows). Note the dense cellularity (hemocytosis) in the vicinity of the parasites. Bar, 10 µm, H&E.

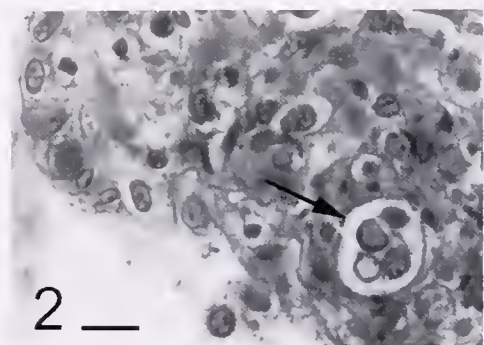


Figure 2. Higher magnification of a cyst of *Perkinsus* sp. trophozoites in the gill tissue of the Manila clam (arrow). Bar, 10 µm, H&E.

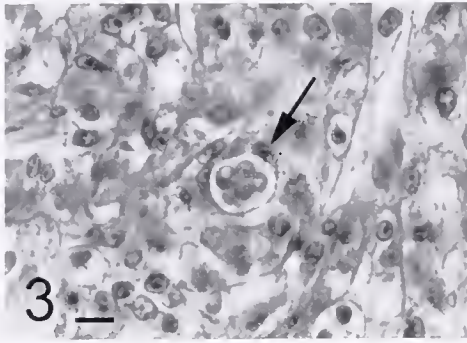


Figure 3. Cyst of *Perkinsus* sp. trophozoites encapsulated by a hemocyte within the gill of a Manila clam. Bar, 10 μ m, H&E.

quently in the mantle and labial palps. Parasites were often associated with tissue hemocytosis (Fig. 1) and occurred as single or multiple trophozoites (Fig. 2). In severe infections, the parasites were more abundantly distributed in the tissues, including the vascular sinuses around the digestive diverticula. Broad areas of the subepithelial connective tissues were composed of solid masses of parasite cysts in the most severe infections. In many cases, the parasites were contained within a thin-walled cyst formed by one to several host cells (Fig. 3). Such encapsulations contained up to 10 protozoan cells and associated hemocytosis. The parasites were often characterized by the presence of an eccentric vacuole (Fig. 1 and 3), characteristic of *Perkinsus* sp. trophozoites.

Confirmation of *Perkinsus* sp. by ISH

The genus-*Perkinsus* SSUrRNA gene probe Perksp700DIG demonstrated strong hybridization to *Perkinsus* sp. cells in all of the tissue sections, except those of *P. qugwadi* infecting *P. yessoensis* (Table 1 and Fig. 4A–I). No hybridization to parasite cells of other genera was observed. ISH of parasite cells in tissue sections of infected Korean Manila clams with this genus-*Perkinsus* probe confirmed the genus level affiliation of the parasites in our sample of juvenile Korean Manila clams (Fig. 5).

DISCUSSION

We report the confirmation by ISH assays and histology of *Perkinsus* sp. infections in Manila clam seed proposed for the introduction into Mexican waters and the subsequent transport to growout sites on the Pacific coast of the United States. This is the first confirmation by a molecular diagnostic probe of *Perkinsus* sp. infection of Korean Manila clams. As a result of these findings, the plan for importation of these clams was rejected by the shellfish producer, and no Korean seed clams were imported to the west coasts of Mexico or the United States. However, the ready availability of such infected seed clams from Korean or Japanese producers requires vigilance to ensure that no such importations take place into areas that are free of the pathogen, such as the west coasts of North and Central America. Reports of lethal *Perkinsus* sp. infections in European and eastern Asian Manila clams from latitudes as far north as that of northern Oregon, confirm the high

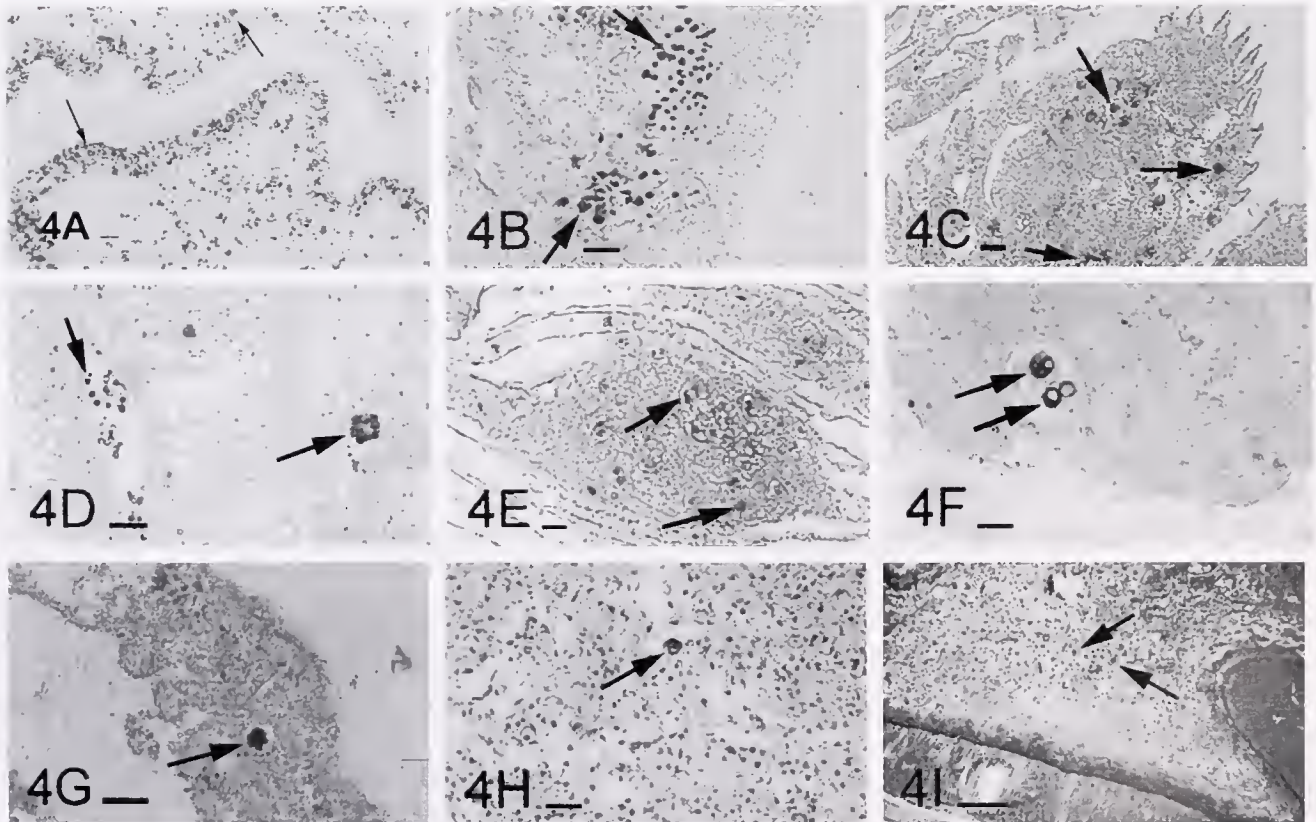


Figure 4. Tissue sections of host tissues reacted with the genus-*Perkinsus* probe Perksp700 by ISH. Positively stained *Perkinsus* sp. parasites are shown by arrows. (A) *P. marinus* in *C. virginica* intestine (bar, 10 μ m). (B) *Perkinsus* sp. in *C. pacificus* (bar, 10 μ m). (C) *P. atlanticus* in *R. decussatus* (bar, 10 μ m). (D) *P. olseni* in *H. laevigata* gill and mantle (bar, 25 μ m). (E) *Perkinsus* sp. in *M. balthica* (bar 25 μ m). (F) *Perkinsus* sp. in Japanese *V. philippinarum* (bar, 10 μ m). (G) *P. chesapeakei* in *M. arcuaria* (bar 10 μ m). (H) *P. mediterraneus* n. sp. in *O. edulis* (bar, 10 μ m). (I) *P. qugwadi* in *P. yessoensis* (no hybridization observed) (bar, 25 μ m).

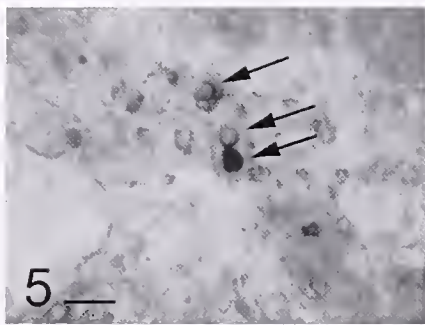


Figure 5. Tissue section of Korean Manila clam reacted with genus-*Perkinsus* probe Perks700DIG by ISH. Positively stained *Perkinsus* sp. parasites are shown by arrows. Bar, 10 µm.

likelihood that such infections, if introduced, could persist and be transmitted, with damaging results to both wild and cultured clam stocks along the Pacific coasts of North and Central America.

This study demonstrated that infection prevalence in seed clams ranging from 16 to 32 mm SL can be nearly 100% and that high parasite intensities cause significant histologic damage to the organs of infected clams, particularly the gills.

Choi and Park (1997) studied five species of Korean clams for infections by *Perkinsus* sp. using Ray's fluid thioglycollate medium (Ray 1966) and found infected Manila clams along the south coast of Korea. While no infection occurred in clams of <15 mm SL, nearly 100% infection prevalence occurred in clams of >20 mm SL. Park et al. (1999) reported mass mortality of Manila clams along the west and south coasts of Korea over a period of several years, which was associated with *Perkinsus* sp. infections. They reported 100% infection prevalence in 142 clams from Komsoe Bay on the west coast of Korea with moderately severe mean parasite intensities of 2.87 based on the infection intensity scale of Choi et al. (1989). A negative correlation was found between the intensity of *Perkinsus* sp. infections and the clam condition index, while clam size was positively correlated with infection intensity.

Maeno et al. (1999) reported *Perkinsus* sp. parasites in Manila clams from an inner bay of the western part of Japan in April 1998, using genus-*Perkinsus*-specific antibodies. These authors concluded that the parasites were *Perkinsus* sp. based on a positive reaction with both single and clustered trophozoites. Hamaguchi et al. (1998) have reported the first detection of *Perkinsus* sp. in Japanese Manila clams. Anecdotal information that we received from the Korean supplier of the seed clams and their Japanese customers indicated that the Manila clam seed had been transported from the Korean source to Japan for at least 20 y with no unusual mortalities or loss of growth reported. This anecdotal re-

port and the multiple reports of the *Perkinsus* sp. parasite occurring about 1997 or 1998 in Japan and Korea suggest that it could have been a new introduction to the Korean clams, as well as the Japanese clams, at about this time.

Manila clams and other bivalve species from Europe reportedly have been infected with *Perkinsus* sp., as follows: *P. atlanticus* from the Mediterranean coast of Spain (region of the Ebro Delta, Tarragona, Spain) infected *R. philippinarum* (Sagrasta et al. 1996); Manila clams from the Lagoon of Venice in northeast Italy infected with a *Perkinsus* sp. (DaRos et al. 1998); and *P. atlanticus* infected the carpet shell clam (*R. decussatus*) from European locations (Ordas et al. 2000). Villalba et al. (2000) reported a significant correlation between the SL of *R. decussatus* and *P. atlanticus* infection intensity. No clams of <20 mm SL were infected, and the highest seasonal parasite intensities occurred in spring and late summer to early autumn.

The relationship of *Perkinsus* sp. in European waters to the *Perkinsus* sp. found in Korea and Japan is unknown at this time. Nonetheless, this and other studies cited in this report indicate the presence of this damaging parasite in Korean and Japanese Manila clams, confirmed first in this study by histology and then definitively by the *Perkinsus* sp.-specific probe presented for the first time in this article. This knowledge can be used to prevent the unintentional introduction of this parasite to west coast of North and Central America. We urge that the science presented in this article be applied by shellfish growers, and by natural resource and conservation managers to prevent such a damaging introduction.

ACKNOWLEDGMENTS

N. A. Stokes, K. L. Hudson, K. Apakupakul, and R. M. Hamilton provided expert technical assistance in the performance of ISH assays. *Perkinsus* sp.-infected mollusc histologic samples were generously provided by C. Azevedo, S. M. Bower, E. M. Bureson, C. L. Goggin, F. G. Kern, and Y. Maeno. Parasitic dinoflagellate-infected crustacean tissue samples were provided by J. D. Shields and G. D. Stentiford. This work was supported in part by National Oceanic and Atmospheric Administration (NOAA) Sea Grant funding of project NA86RG0037 to CFD. This work is also a result of research sponsored in part by NOAA Office of Sea Grant, U.S. Department of Commerce, under grant No. NA96RG0025 to the Virginia Graduate Marine Science Consortium and the Virginia Sea Grant College Program, and under grant No. NA016RG2207 to the Maryland Graduate Marine Science Consortium and the Maryland Sea Grant College Program. The U.S. Government is authorized to produce and distribute reprints for governmental purposes, notwithstanding any copyright notation that may appear hereon. VIMS contribution #2575.

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FILTRATION AND RESPIRATION RATES OF THE ELONGATE SUNSET CLAM *GARI ELONGATA* LAMARCK 1818 UNDER NATURAL LIGHT CONDITIONS

ANNABELLE G. C. DEL NORTE-CAMPOS*

Marine Biology Laboratory, Division of Biological Sciences, College of Arts & Sciences, University of the Philippines in the Visayas, Miagao, Iloilo 5023, Philippines

ABSTRACT This study is a first step in evaluating the potential of the suspension-feeding clam *Gari elongata* as a biofilter in lake/pond polyculture systems. Filtration and respiration rates across all sizes of *G. elongata* were measured in the laboratory under natural light conditions. Filtration rates (F) were determined in a batch system at four concentrations (10 , 25 , 50 , and 100×10^4 cells mL^{-1}) of the phytoflagellate *Isochrysis galbana*. Filtration rates were higher at lower concentrations but decreased with the highest concentration, indicating a possible overloading of the gill sorting mechanism. For the given conditions, mean F at $45.1 \text{ l h}^{-1} \text{ g}^{-1}$ was considered optimal at 25×10^4 cells mL^{-1} . This value proves to be the highest F value reported so far, for bivalves. The results further qualify the species as a biofilter in fresh- to brackishwater polyculture setups. F values were further observed to decrease with body weight, although there was a wide variation. The $F:W$ exponent computed for the species was inconsistent with reported trends, which may be due to either light conditions or algal cell size. There was no increase in the species' respiration rate (R) with feeding. For unfed clams, R decreased with size, but there was high variation, and the relationship was not significant. At optimum algal concentration, R per unit body mass for fed clams, decreased with size, and the computed $R:W$ exponent value points to a less rapid increase in size-respiration rate than most other bivalves.

KEY WORDS: filtration rates, respiration rates, *Gari*, body size, algal concentration, physiology

INTRODUCTION

Intensification in aquaculture has become a choice option to ensure food security in the face of an ever-increasing population and parallel demand for fishery products. Hence, there is a continuous effort to increase production in fishponds, pens and cages (Briggs & Funge-Smith 1994, Funge-Smith & Briggs 1998). Along with this effort, however, are emergent concerns relating to the environmental impact of aquaculture, specifically the increased organic loading from excess feeds and wastes, observed in freshwater lakes and coastal habitats.

In the light of these concerns, polyculture with biofilters, or co-rearing invertebrates and aquatic macrophytes with fish and/or crustaceans, may serve as the solution (e.g., Shpigel & Fridman 1990, Shpigel & Blaylock 1991, Shpigel et al. 1993, Ahlgren 1998). These filter- (e.g., bivalves) or deposit- (e.g., sea cucumbers) feeding invertebrates and seaweeds can help maintain water quality by using up excess bottom-accumulated organic matter (e.g., excess feeds, fecal material) and suspended dissolved nutrients, thereby converting these to usable or harvestable biomass.

Although polyculture in marine waters has received ample attention, using bivalves in freshwater systems is a relatively new topic. A possible biofilter species is the filter-feeding psammobiid clam *Gari elongata* Lamarck, a lesser-known bivalve species that burrows in shallow, freshwater to brackish (0 – 15 ‰) waters in Panay island, located in the west central Philippines (del Norte-Campos, submitted). It is characterized as having brownish black, elongated, and moderately thick shells. This species may be a potential polyculture species in tilapia cage culture (lakes) and/or carp and catfish ponds. It is harvested and sold for food in local markets, albeit at low prices and quantities. Although abundant, the species has received little scientific attention, possibly because they closely resemble the more popular and studied, brown mussel (*Modiolus metcalfei*) in appearance.

This study on *G. elongata* had the overall goal to quantify the components of the species' energy budget, $C = f + U + P + R$ (modified from Windell 1978), where the energy of the food consumed (C) is apportioned to the energy lost in the form of feces (f), urine or excreta (U), and respiration or metabolic cost (R) and gained in the form of tissue growth (P). Food consumption (C) in filter-feeding bivalves is a function of filtration (F) and absorption (A).

The specific objectives of the present work were to measure the 1) filtration rates (F) of the species, in terms of body size and algal concentrations and 2) respiration rates (R) for unfed and fed individuals of various size classes.

MATERIALS AND METHODS

Study Area and Laboratory Experiments

The clams used for this study were collected by hand from rice farm irrigation canals in Barotac Viejo, Iloilo province, eastern Panay Island in west central Philippines (Fig. 1). Water depth in the area was around 1 m, with salinity ranging from 0 to 10 ppt. Bottom substrate was muddy to silty. These canals drain into the Alacaygan River, which empties into the Banate Bay. The clams were brought to the laboratory and acclimated for at least 3 days prior to the start of the experiments. Although the species tolerates salinities of up to 15 ppt (del Norte-Campos, submitted for publication), all experiments were conducted using freshwater, with water temperatures ranging from 26 to 30°C .

Morphometrics

Shell length (in mm) was measured with the use of a vernier caliper. Filtration and respiration rates were determined for six size classes: 10 – 19.99 , 20 – 29.99 , 30 – 39.99 , 40 – 49.99 , 50 – 59.99 , and 60 – 69.99 mm. Wet and dry weights (g) of individuals from across all size ranges were taken. Soft tissue was dried at 60°C to constant weight. The relationship between shell length and dry soft tissue weight was derived through regression analysis. For each size

*Corresponding author. E-mail: willbell@iloilo.net

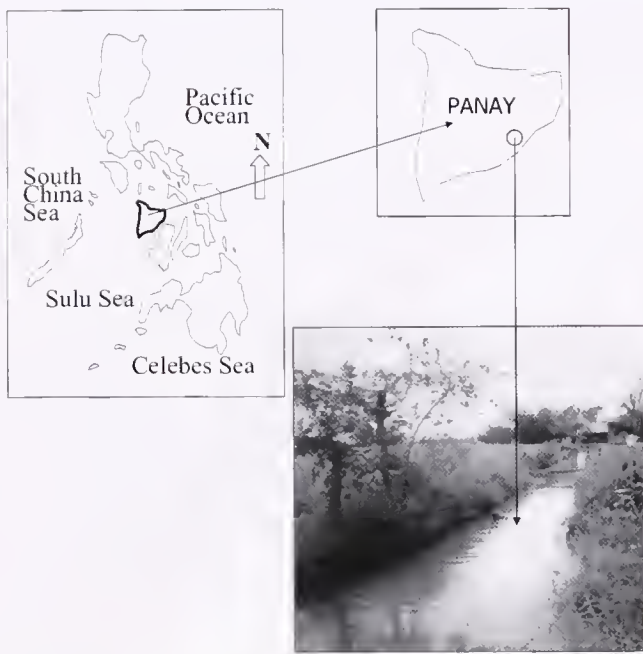


Figure 1. Site of collection of the clam *Gari elongata*: irrigation canal (approx. 0.5 m in depth) in Barotac Viejo, Iloilo, Panay Island, The Philippines.

class, three trials (replicates) were conducted, with each trial consisting of three individuals.

Microalgae

The phytoflagellate *Isochrysis* aff. *galbana* Tahitian (*T-Iso*), harvested during its logarithmic phase of growth, was used as food. The species was selected for its relatively small size (mean cell diameter approx. 4 μm), and nutritional value, that it is therefore, widely used in mariculture (Taylor et al. 1997, Brown et al. 1999, Phatarpekar et al. 2000).

Filtration Rates

Four microalgal concentration levels were tested: 10, 25, 50, and 100×10^4 cells mL^{-1} . These values correspond to levels that are normally encountered in aquaculture ponds (Duerr et al. 1998). The desired concentration and volume was obtained through dilution.

The volume of water that each bivalve filtered of particulate material (F , L h^{-1} ; or the indirect method of Jørgensen 1990) was determined from separate containers (V approx. 8 L) holding the bivalves. Water sampling was done every thirty minutes during a 3-h experiment. A 5-mL sample was collected each time from the center of the containers and immediately fixed with two drops of Lugol's solution to prevent further reproduction of the algal cells. The density of the algal population in every sample was measured by direct algal cell count using a hemocytometer and a microscope. Three replicate counts were made for each sample. A parallel control set-up showed no significant change in algal cell densities during the 3-h observation period.

For each clam, the filtration rate was determined using the formula $F = Vr$, where r is the rate constant (h^{-1}), or the negative of the slope obtained from regressing $\ln C$ or cell concentration (mL) against time (h) and V is volume of the diluted algal suspen-

sion containing the clams. Because filtration rates were measured in a batch system where algal concentrations do not remain constant, only the descending limb of the curve was included in the determination of the slope, that is, the flatter portion (asymptote) was excluded. Derived filtration rates were compared for the four algal cell concentrations, and based on this, an optimal cell concentration was chosen. For the optimum concentration, the relationship between F and dry wt (g) was derived, and compared with reported values. To show the relationship of filtration rates with size, filtration rates were expressed in terms of the dry weight of the organisms ($\text{L h}^{-1} \text{g}^{-1}$) and plotted against the corresponding dry weights (g) of the organisms.

Respiration Rates

Respiration rates were measured for both unfed (ration = 0; starved for 24 h) and fed clams. The latter were also given *T-Iso* at the observed optimal algal concentration in a holding container for at least 15 min. prior to respiration measurements. For both unfed and fed clams, three individuals per size class were placed in a sealed transparent Plexiglas chamber ($15 \times 15 \times 15$ cm, or 3.4-L capacity). Oxygen concentration in each chamber was measured at 30-s intervals using a YSI DO meter. A parallel control with no clams was run to test the stability of the chamber. Respiration rate (R , $\text{mL O}_2 \text{ ind.}^{-1} \text{ h}^{-1}$) was determined as the negative of the slope of $\ln \text{O}_2$ concentration vs. time (h). Initial data plots served as bases to decide the time series portion that was included. The relationship between R and dry wt was likewise derived, and compared with reported values in the literature. To show the effect of size as in above, respiration rates were also expressed in terms of the dry weight of the organisms ($\text{mL O}_2 \text{ h}^{-1} \text{g}^{-1}$) and plotted against the dry weights (g) of the organisms.

RESULTS

The range of sizes used in the experiments was 11.8–67.9 mm, corresponding to 0.008–1.298 g dry weight. Filtration rates (F) for the algal concentrations of 10, 25, and 50×10^4 cells mL^{-1} averaged for all sizes were found to be higher (81.3, 45.1, and 68.5 $\text{L h}^{-1} \text{g}^{-1}$ resp.) and lowest (25.9 $\text{L h}^{-1} \text{g}^{-1}$) at the highest algal concentration of 100×10^4 cells mL^{-1} (Fig. 2). Although the differences were found to be statistically significant, the optimum algal cell concentration selected was 25×10^4 cells mL^{-1} , as this was the median of the range of concentrations where F values were higher. This would further be the logical choice considering that among all four concentrations, it was with this concentration that the F was

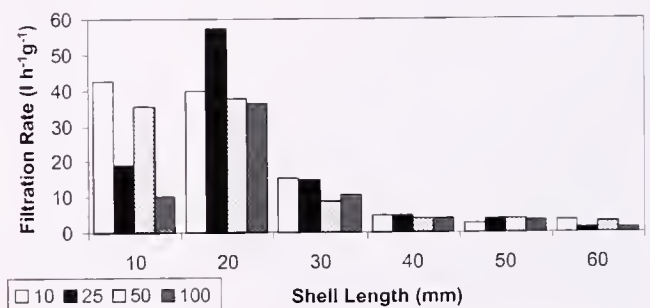


Figure 2. Filtration rate ($\text{L h}^{-1} \text{g}^{-1}$) of *G. elongata* versus shell length (mm) under different algal concentrations (10, 25, 50, and 100×10^4 cells mL^{-1}). Values from the 10-mm size class are $\times 10^{-1}$.

highest for four size categories (20, 30, 40, and 50 mm) out of the six (excluding 10- and 60-mm size classes; Fig. 2).

Filtration rates ($\text{l h}^{-1} \text{g}^{-1}$) plotted against dry weight (g) was shown to decrease with size with the slope equivalent to -1.1196 and $r^2 = 0.9723$ (Fig. 3). The regression was found to be significant ($P < 0.01$), i.e. the intercept and slope are significantly different from 0.

Respiration rates in terms of weight ($\text{mL O}_2 \text{h}^{-1} \text{g}^{-1}$) plotted for both unfed (Fig. 4) and fed (Fig. 5) clams were observed to decrease with size. The relationship for the latter was significant, with a slope of -0.6712 and $r^2 = 0.848$. The mean computed rate for unfed clams were $0.61 \text{ mL O}_2 \text{ind}^{-1} \text{h}^{-1}$, whereas at optimal algal concentration values ranged from 0.11 to $0.96 \text{ mL O}_2 \text{ind}^{-1} \text{h}^{-1}$.

DISCUSSION

The observed initial increase and subsequent decrease in filtration rates of *G. elongata* with increasing cell concentration (Fig. 2), is similar to several results on different lamellibranch bivalves (e.g., Winter 1970, Tenore & Dunstan 1973, Schulte 1975, Gerder 1983) indicating that filter feeding is affected by cell concentration. This has likewise been observed even for bivalve larval stages. In the oyster *Ostrea edulis* for example, ingested ration was correlated with algal cell concentration, whereby further increases in the latter failed to support higher ingestion rates and faster growth rates (Beiras & Perez Camacho 1994). It is apparent therefore, that bivalves regulate the amount of water filtered in relation to food concentration. The increase in filtration rates would suggest sub optimal algal concentrations, whereas inhibition of filtering activity would indicate a possible overloading of the gill sorting mechanism at higher cell concentrations (50 to 100×10^4 cells mL^{-1} ; Winter 1978). This adjustment behavior, which could possibly be linked to satiety, was further observed to be coupled with mucus secretion and pseudofeces production at high cell concentrations, meaning that the digestive capacity of the clams has been exceeded (Riisgård & Mohlenberg 1979).

The filtration rates measured for this species proved to be the highest of values reported for bivalves (Table 1). As shown in Table 1, literature values for tropical and subtropical species measured for a variety of conditions, range from 1.9 to $11.5 \text{ l h}^{-1} \text{g}^{-1}$, with the highest values reported for pearl oysters *Pinctada margaritifera* and *P. maxima* (Yukihira et al. 1998). The filtration rates of temperate bivalves on the other hand, were reported to range from 0.6 to 14.7 , with the highest values reported likewise for the pearl oyster *P. fucata martensii* (Numaguchi 1994). The present values refer however, to smaller clams, compared with the bigger sizes of pearl oysters (36 – 185 mm) used by Yukihira et al. (1998).

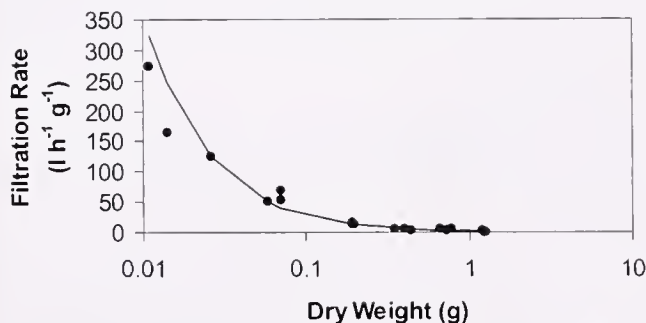


Figure 3. Filtration rate ($\text{l h}^{-1} \text{g}^{-1}$) of *G. Elongata* versus dry weight (g), $F = 2.0853 \text{ DW}^{-1.1196}$; $r^2 = 0.9723$, $n = 18$. X-axis in log scale.

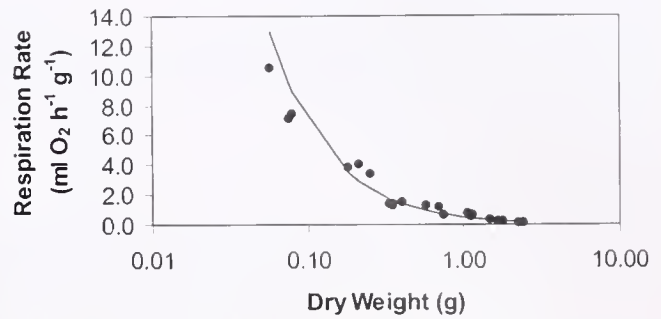


Figure 4. Respiration rate ($\text{mL O}_2 \text{h}^{-1} \text{g}^{-1}$) of *G. elongata* versus dry weight (g) for unfed individuals ($R = 0.5062 \text{ DW}^{-1.1544}$; $r^2 = 0.955$, $n = 21$). X-axis in log scale.

Thus, the higher filtration rates of smaller sizes explain the higher mean filtration rates.

Aside from size, the morphology and habit of the species are likewise factors to consider in comparing filtering capacities. According to Yukihira et al. (1998), to maximize growth in pearl oysters, strong and fast water currents is a requisite, especially because they occur in oligotrophic waters. Filtration rates of *G. elongata* are higher compared with those reported for giant clams (0.1 to 3.7 l h^{-1} , with lowest values measured for *Tridacna derasa* and *T. devoraa*; Klumpp & Lucas 1994). Giant clams, however, are both auto- and heterotrophic, and that despite these lower rates, they achieve large sizes by supplementing suspension feeding by translocation of photosynthates from symbiotic zooxanthellae (Lucas 1994). The high filtration rates of *G. elongata* show that the species is well adapted to its environment, characterized by fast-flowing water currents. A mean growth rate of $0.13 \pm 0.06 \text{ mm day}^{-1}$ was estimated over a period of 1 year (del Norte-Campos, submitted). Thus for this species, filtering at higher rates could well support high growth rates typical of tropical short-lived species. Furthermore, this ability to filter at higher rates also qualifies it as a biofilter species in fresh- to brackishwater polyculture set-ups.

In relation, the clearance rates measured for phytoplankton taxa and cyanobacteria filaments in the likewise freshwater (lake) bivalve *Dreissena polymorpha* were in contrast, reported to be lower, ranging from 0.2 to 0.34 L h^{-1} (Horgan & Mills 1997). The latter may thus be possibly due to the species' adaptation to a habitat with lesser turbulence.

Furthermore, Jørgensen (1990, 1996) concluded that the capacity of water processing in bivalves is evolutionarily adapted to the

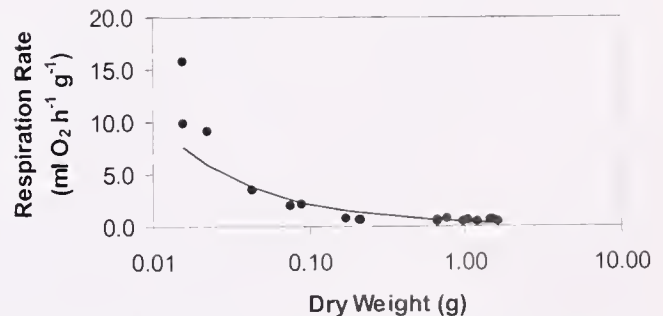


Figure 5. Respiration rate ($\text{mL O}_2 \text{h}^{-1} \text{g}^{-1}$) of *G. elongata* versus dry weight (g) for unfed individuals ($R = 0.4620 \text{ DW}^{-0.6712}$; $r^2 = 0.848$, $n = 18$). X-axis in log scale.

TABLE 1.

Filtration rates (F , $l\ h^{-1}$) and respiration rates (R $ml\ O_2\ h^{-1}$) for various bivalve species size-standardized for 1 g dry soft tissue weight (*exceptions indicated under Conditions) (+ as cited in Yukihiro et al 1998).

Species	F	R	Conditions	Literature
Tropical/Subtropical bivalves				
Clams				
<i>Gari elongata</i>	45.1	0.11–0.96	Fed with Tahitian <i>Isochrysis galbana</i> (T-iso) at 250 T cells ml^{-1} , 28 °C; for size range 11.8–67.9 mm	Present study
		0.81	Unfed	
<i>Arca zebra</i>	3.13	0.30	*0.82 g dry tissue wt, <i>Chaetoceros calcitrans</i> (15 T cells ml^{-1}), 30 ± 0.5 °C	+Widdows et al (1990)
Pearl oysters				
<i>Pinctada imbricata</i>	5.2		Natural particles (3.1 $mg\ l^{-1}$), 22 °C	+Ward & MacDonald (1996)
<i>P. margaritifera</i>	11.5	1.04	T-iso (~5T cells ml^{-1}), 28 °C; 36–152 mm SH	Yukihiro et al (1998)
		1.05	*Oyster of 100 g total wt, 28 °C	+Shigiyama & Tomori (1988)
<i>P. margaritifera</i> var. <i>cumingi</i>		0.34	Routine rate, <i>C. calcitrans</i> and T-Iso (50 T cells ml^{-1})	+Stiger (1993)
<i>P. maxima</i>	11.5	0.86	T-iso (~5T cells ml^{-1}), 28 °C; 37–185 mm SH	Yukihiro et al (1998)
Scallops				
<i>Amusium pleuronectes</i>	6.8		<i>I. galbana</i> (60 T cells ml^{-1}), 28 °C	+Rise et al (1994)
Mussels				
<i>Perna perna</i>	2.55	0.41	<i>Thalassiosira weissflogii</i> and natural particles (3 $mg\ l^{-1}$), 15 °C	+Van Erkom Schurink & Griffiths (1992)
<i>P. viridis</i>	2.3	0.43	Natural particles (POM: 1.3 $mg\ l^{-1}$), 28 °C	+Krishnakumar et al (1990)
Giant clams				
<i>Hippopus hippopus</i>	0.52	0.10	Natural particles or <i>Dunaliella terilolacta</i> , 24–27 °C	+Klamm & Griffiths (1994)
<i>Tridacna crocea</i>	0.58	0.61		
<i>T. gigas</i>	3.68	1.06		
<i>T. squamosa</i>	0.32	0.48		
<i>T. derasa</i>	0.12	0.16	*Size-standardized for 1 g wet tissue wt, 20–26 °C	+Klamm & Lucas (1994)
<i>T. levorosa</i>	0.14	0.29		
Temperate bivalves				
Clams				
<i>Mercenaria mercenaria</i>	2.6		Dyes as suspension, 18–20 °C	+Odughlan & Ansell (1964)
<i>Rangia cuneata</i>	0.56	0.16	<i>I. galbana</i> and <i>T. fluviatilis</i> mixed, 21.1 °C	+Hartwell et al (1991)
Oysters				
<i>Crassostrea gigas</i>	3.65	0.54	Natural particles (100 $mg\ l^{-1}$), 15–18 °C	+Barille et al (1997)
<i>C. virginica</i>	2.55	0.24	<i>I. galbana</i> and <i>Thalassiosira fluviatilis</i> mixed, 21.1 °C	+Hartwell et al (1991)
<i>Ostrea edulis</i>		0.36	*1 g AFDW, 5 °C	+Rodhouse (1978)

concentrations of suspended food, primarily phytoplankton. Initial experiments showed that *G. elongata* had higher filtration rates under dark feeding conditions (Piñosa, unpublished observations), and this could perhaps be explained by the natural water conditions (silty) in the clam's biotope. Difference in day and night feeding was similarly observed in the zebra mussel *D. polymorpha* inhabiting freshwater lakes (Horgan & Mills 1997).

The decrease in filtration rate per unit body mass with increase in size (Fig. 3) is consistent with reported trends (e.g., Widdows 1978). However, the computed $F:W$ exponent value (2.0753) does not fall within the range (0.3 to 0.8, mean = 0.62) reported for several filter-feeding bivalves (Bayne & Newell 1983). This may be attributed to the wide variability in the results. However at the same time, there are also reports regarding the absence of relationship between filtering activity and sizes (e.g., Horgan & Mills 1997). The present results may likewise, be due to less preferred light conditions used in the experiments. Preliminary work on the species' filtration rates also suggested that the highest preference was for the smallest algal size, that is, *Nannochloropsis* sp. (Piñosa, unpublished observations). It may be possible then, that the cell diameter of *T-Iso* is still somewhat large for *G. elongata*.

Reported respiration rate values (Table 1) range from 0.3 to 1.05 $ml\ O_2\ h^{-1}g^{-1}$ in tropical and subtropical bivalves, 0.1 to 1.06 $ml\ O_2\ h^{-1}g^{-1}$ for giant clams, and 0.06 to 0.93 $ml\ O_2\ h^{-1}g^{-1}$ for temperate bivalves. Thus, the values for *G. elongata* of 0.11–0.96 $ml\ O_2\ h^{-1}$ fall within the reported range. Bayne & Newell (1983) gave about 0.7 (range 0.4 to 1.0) as the mean value of allometric exponents for the $R:W$ relationship for a variety of marine mollusks. Thus the value (0.4620; Fig. 5) for fed *G. elongata* from this study fall in the lower range, which means that there is less rapid increase in the size-specific metabolic rate of this species than most other bivalves.

The study showed that the species is suitable for use as a biofilter organism. This could further be verified by conducting experiments in polyculture set-ups, both under laboratory and field conditions.

ACKNOWLEDGMENTS

This study received financial support from the University of the Philippines (UP) System-Creative and Research Scholarship Pro-

gram (CRSP). M.A. Tad-y provided laboratory assistance. W. L. Campos gave suggestions and help in the preparation of the manuscript. Laboratory space was kindly provided by the UPV-Institute

of Aquaculture Hatchery, kindly facilitated by S.S. Garibay. The author thanks the reviewers for their helpful suggestions and comments.

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EL NIÑO AND LA NIÑA EFFECTS ON REPRODUCTIVE CYCLE OF THE PEARL OYSTER *PINCTADA MAZATLANICA* (HANLEY, 1856) (Pteriidae) AT ISLA ESPÍRITU SANTO IN THE GULF OF CALIFORNIA

J. ANGEL GARCIA-CUELLAR,^{1,2,*} FEDERICO GARCIA-DOMINGUEZ,¹
DANIEL LLUCH-BELDA,¹ AND SERGIO HERNÁNDEZ-VAZQUEZ²

¹Centro Interdisciplinario de Ciencias Marinas (CICIMAR), Instituto Politécnico Nacional, La Paz, B.C.S. 23000, Mexico; ²Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, B.C.S. 23000, Mexico

ABSTRACT The impact of El Niño (1997–1998) and La Niña (1998–1999) on the reproductive cycle of the pearl oyster *Pinctada mazatlanica* was studied by histologic observation of gonads, determination of the conditioning index, and oocyte monthly mean diameter, which are related to sea surface temperature and availability of food. Available food was determined by comparison between seasonal and a standard concentration of chlorophyll *a*. Oyster specimens were collected from Isla Espíritu Santo in the Gulf of California from May 1997 to November 1999. A direct relationship of spawning to temperature was observed from July to October. Concentrations of chlorophyll *a* were related to the stage of gonad development. The reproductive process was related to the conditioned index. Reproductive activity during El Niño was longer, with spawning in July to November 1997 and July to October 1998, and more intensive (in terms of population spawning frequency) than that during the La Niña event, when the spawning occurred during August to November 1999.

KEY WORDS: El Niño, La Niña, pearl oyster, *Pinctada mazatlanica*, reproduction

INTRODUCTION

The pearl oyster *Pinctada mazatlanica* is distributed from the west coast of the Baja California Peninsula south to Peru, including the southern part of the Gulf of California and islands around the Peninsula (Keen 1971, Martínez 1983). During the past century, the quality of the pearls produced by this species supported an important pearl fishery in the Gulf of California, which was the incentive for colonization of Baja California Sur (Cariño & Caceres-Martínez 1990, Monteforte & Cariño 1992). The fishery was closed by the Mexican government in 1938 because of overfishing (Keen 1971, Monteforte 1990, Sevilla 1969).

The pearl oyster *P. mazatlanica* is considered a potential and feasible resource to be developed by mariculture (Monteforte 1990). Thus, its reproductive biology is a relevant study subject. In Mexico, some studies have been performed on cultured specimens (Díaz 1972, García-Gasca 1992, Gaytan et al. 1993, Saucedo-Lastra & Monteforte 1997) and on wild populations (Monteforte 1991, Moreno & Moreno 1994, Wrigth 1997). Studies of reproduction in wild populations from Bahía de La Paz were performed by Sevilla (1969) and García-Domínguez et al. (1996); from Guaymas, Sonora, by Arizmendi-Castillo (1996); and from the Golfo de Nicoya, Costa Rica, by Solano-López et al. (1997). In these works, there were no reported relationships and effects on reproduction in *P. mazatlanica* by El Niño and La Niña events. This study describes the effects on the reproductive cycle of *P. mazatlanica* through changes in sea surface temperature and availability of food due to the El Niño and La Niña climatic events.

MATERIALS AND METHODS

Specimens were collected monthly and randomly from May 1997 to November 1999 (except January 1999) in a wild population located near Isla Espíritu Santo, B.C.S. (Fig. 1), from a depth of 4 to 6 m by personnel using scuba gear. A total of 523 organisms were collected, ranging from 20.4 to 228.3 mm shell length

(mean, 119.7; SD, 27.6) and 21.2 to 167 mm shell height (mean, 113.9; SD, 23). Before dissection, the shell length and height were measured with calipers (0.01 mm resolution); subsequently, the wet weight was obtained by electronic scales (0.01 g resolution). The visceral mass (including the gonad) was fixed in a solution of 10% formalin prepared with seawater, dehydrated in an alcohol series dilution, and embedded in paraffin (Luna 1968). Sections, in a frontal and dorsal plane, 5- to 6- μ m thick, were made and stained with Harris' hematoxylin and eosin (Luna 1968).

The diameter of at least 100 oocytes was measured using digitized images from the histologic preparations (Sigma Scan Pro4 software program), in each of seven females per month selected randomly. The measurements were made along the longest axis in the oocytes sectioned through the nucleus containing clearly visible nucleoli. From this data, mean size and standard deviation were obtained. The specimens with few measurable oocytes and extensive phagocytosis were not considered, following the criteria of Grant and Tyler (1983a, 1983b).

Sex was determined by microscopic analysis, and the percentage of each sex was obtained. The sex ratio was determined and examined for deviation from the expected ratio of 1:1 by chi-square (χ^2) analyses. Condition index (CI), using the formula proposed by Ricker (quoted by Bolger & Connolly 1989), was selected because it takes into account those cases in which growth is not isometric, as occurs in *P. mazatlanica*, and could describe in the best way the general condition variations and their relationship with the reproductive event:

$$CI = PH/L^b \times 1000$$

where CI was determined by dividing the visceral mass wet weight (PH) by the anteroposterior length (mm) and powered by *b* (value from the slope, which resulted from the power correlation from both variables). We obtained CI means for each monthly sampling.

Sea surface temperature (SST) values were obtained by Coastal Zone Color Scanner (CZCS) from NOAA and chlorophyll *a* concentrations from the SeaWiFS Project, NASA/Goddard Space Flight Center and ORBIMAGE. Anomalies were estimated as de-

*Corresponding author. E-mail: angelbio@prodigy.net.mx

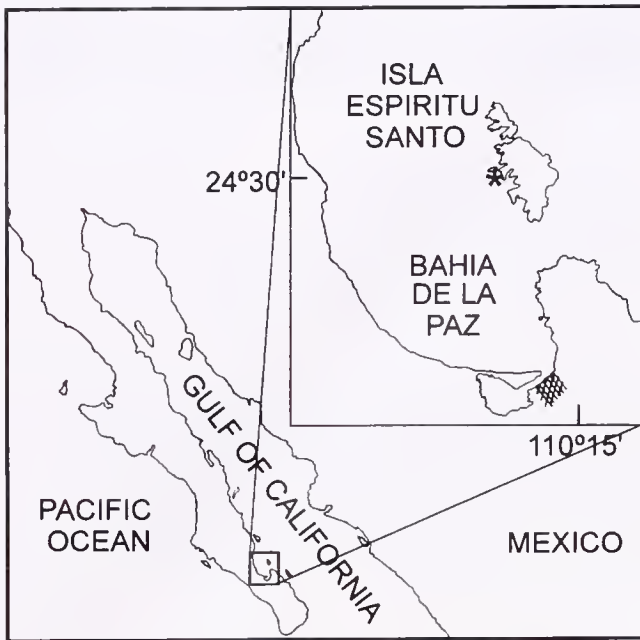


Figure 1. Study area. Isla Espiritu Santo, B.C.S., Mexico.

partures from the averaged monthly cycle; on this basis, we defined the periods influenced by El Niño (May 1997 to October 1998, positive anomalies) and by La Niña (November 1998 to November 1999, negative anomalies).

The condition of the gonad was determined by microscopic

analysis. Each specimen was assigned a specific stage of gametogenic development. Stages were assigned on the basis of the classification described for *P. mazatlanica* by Sevilla (1969) and García-Domínguez et al. (1996). This classification divides the gametogenic cycle into five stages: indifferentiated, developing, ripe, partially spawned, and spent. The relative frequency of each gonad phase was determined. The reproductive period was considered at spawning stage.

RESULTS

Gonad Developmental Stages

The microscopic anatomy from each gonad stage of *P. mazatlanica* did not differ from those described by Sevilla (1969), García-Domínguez et al. (1996), and Saucedo-Lastra and Monteforte (1997).

Reproductive Cycle

The reproductive cycle is shown in Figure 2. We observed that during the El Niño, spawning occurred from July to November 1997 and July to October 1998 (maximum peaks in July to September 1997, and July and August 1998). This period was characterized by the constant appearance of gametogenic activity in developed and ripe stages and relatively poor inactive (indifferentiated stage). The population spawning frequency was 69% mean and 90% maximum. During the La Niña, spawning took place from August to October 1999 (maximum peaks on August to October), predominated by the developed stage and more presence of the inactive stage. The population spawning frequency was 45.6%

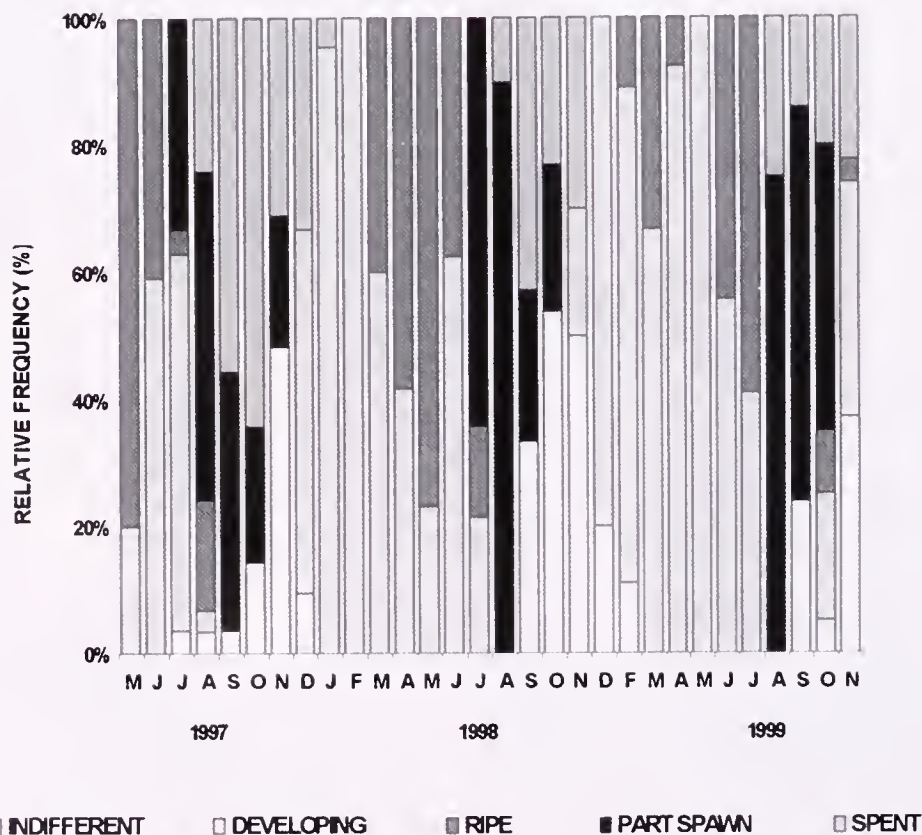


Figure 2. Relative frequency of gonadal stages of *P. mazatlanica* from May 1997 to November 1999 at Isla Espiritu Santo, Gulf of California.

mean and 70% maximum. In both periods, the spawn occurred between 27 and 30°C and was suspended at temperatures under 25°C.

Sex Ratio

Out of 523 specimens sampled, 250 (47.8%) were males, 205 (39.2%) were females, 12 (2.3%) were hermaphroditic, and 56 (10.8%) were undifferentiated. The sex ratio (1.22 male:1 female) did not differ significantly ($P < 0.05$, $n = 455$) from an expected 1:1 ratio.

Analysis of Oocyte Size

Mean oocyte diameters are shown in Figure 3. Maxims were observed during May and June 1997, June 1998, and July 1999. From September to December 1998 and February to March 1999, there were specimens with few or no measurable oocytes. The highest mean oocyte diameter values during El Niño corresponded to the ripe stage (May to June 1997, and April to May 1998), and the lowest mean values corresponded to the spent stage (September to November 1997 and August to October 1998). This relationship did not occur during La Niña.

Condition Index

To validate the CI determination, the height and visceral weight variables were compared. Using a power correlation, we obtained a value of $r = 0.88$ and slope $b = 2.77$ ($P < 0.05$, $n = 523$). The CI values fluctuated between 8–16% and were related to the species reproductive stages, maximum values coinciding with the de-

velopment stage, and the minimum values with the spawn stage (Fig. 4).

Temperature and Anomalies

During the study, the SST varied from 19 to 30.8°C. During El Niño, SSTs varied from 21.6 to 30.8°C, with a maximum anomaly of +2.82°C (January 1998), and during La Niña, from 19 to 28.2°C, with a maximum anomaly of -1.36°C (February 1999). The SSTs and anomalies are recorded in Figure 5.

The relationship between the SST and the relative frequency of spawning are shown in Figure 6. Spawning occurred between the 27 to 30°C interval and was suspended when colder than 25°C. During the El Niño, the highest percentage of population spawned coincided with the highest temperature recordings in August 1997 and August 1998; during La Niña spawning occurred in August 1999, while the highest temperature recorded was during September of 1999.

Photosynthetic Pigment Concentration

Chlorophyll *a* concentrations varied during El Niño from 0.096 to 1.2 mg/m³ and during La Niña from 0.28 to 3.6 mg/m³. In both periods, pigment concentrations were inversely related to the temperature, with the highest concentrations during the winter seasons and the lowest during the spring-summer seasons.

Figure 7 shows the relationship of the reproductive cycle to the pigment concentrations. The highest pigment concentration values were significantly related with the development stage (untransformed data) ($r = 0.75$, $P < 0.05$, $n = 30$) and minimum pigment concentrations values with ripe and spawn stages.

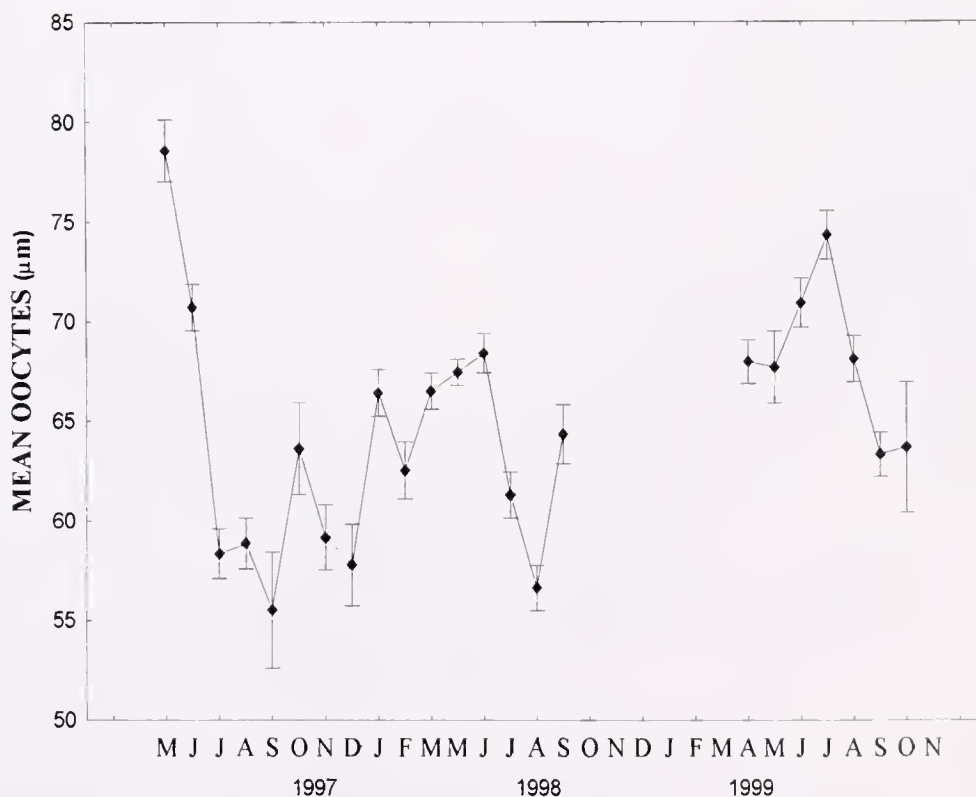


Figure 3. Mean oocyte sizes of *P. mazatlanica* from May 1997 to November 1999 at Isla Espíritu Santo, Gulf of California. Bar = standard deviation.

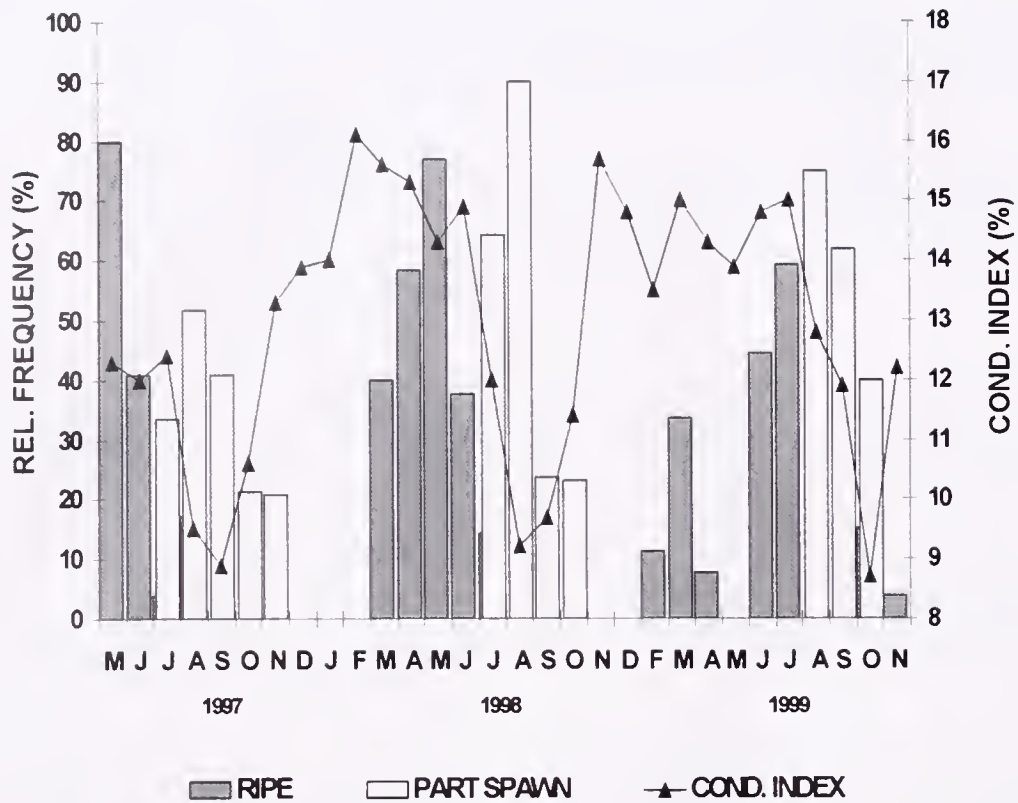


Figure 4. Relationship between the conditioned index and the relative frequency of the ripe and part-spawning stages from May 1997 to November 1999 at Isla Espiritu Santo, Gulf of California.

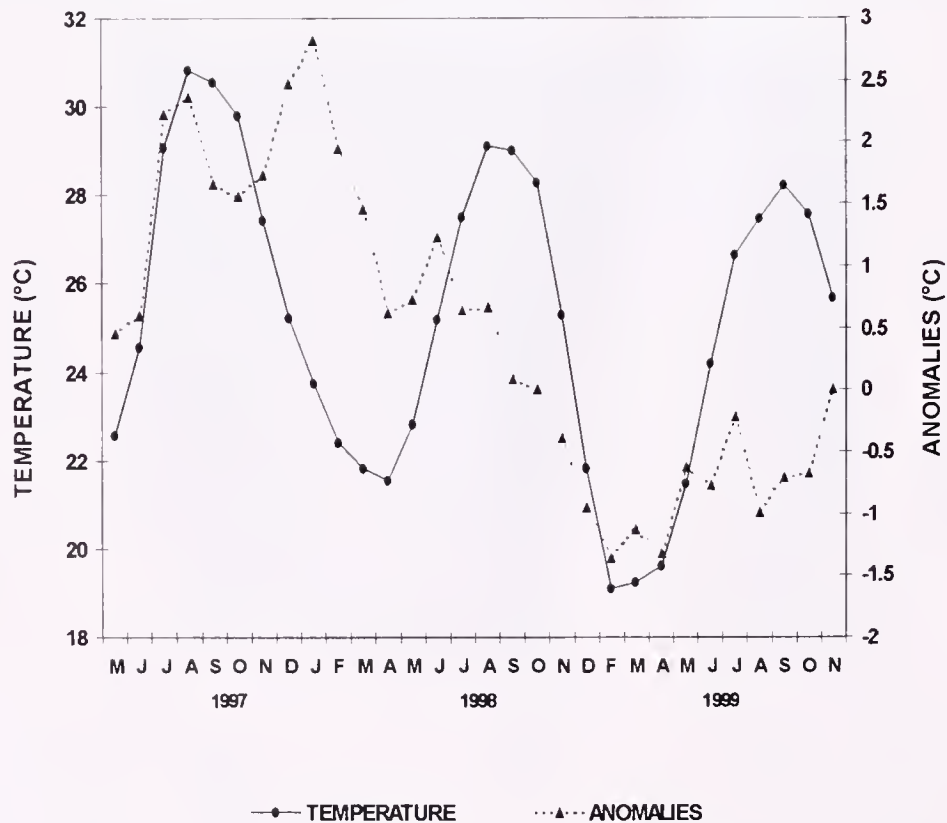


Figure 5. Average monthly sea surface temperature and anomalies from May 1997 to November 1999 at Isla Espiritu Santo, Gulf of California.

DISCUSSION

The description of gonadic cycle stages and their corresponding microscopic anatomy did not differ from those performed for *P. mazatlanica* by diverse authors (García-Domínguez et al. 1996, Saucedo-Lastra & Monteforte 1997, Sevilla 1969).

There were differences related to the gonadic cycle among El Niño and La Niña events. During El Niño, the developing and ripe stages were constants and in equilibrium along the study period whereas the spawning stage was continuous and longer on time duration and more intensive (related to population spawning frequency); the indifferentiated stage was present in low frequency. In contrast, during La Niña the developed stage was predominant along the study period with intermittent and short ripe stage and the reproductive stage was of minor duration and intensity.

Studies performed on *P. mazatlanica* reproduction (García-Domínguez et al. 1996) at Isla Espíritu Santo revealed spawn occurrence during June, July, and October 1992 and April 1993, with 30% mean and 33.3% maximum population spawning frequency whereas Sevilla (1969) in Bahía de La Paz found maximum spawning peaks in August and September 1963, with 30.5% mean and 50% maximum population spawning frequency. On cultured population from Bahía de La Paz, Saucedo-Lastra and Monteforte (1997) found only one maximum spawning peak during September 1992 and 30% maximum population spawning frequency. In Guaymas, Sonora, Mexico, *P. mazatlanica* was reported with a maximum spawning peak in June 1994 and 67% maximum population spawning frequency (Arizmendi-Castillo 1996), and from the Golfo de Nicoya, Costa Rica, two maximum spawning peaks in October 1993 and June 1994, with 31.5% mean population spawning frequency were reported (Solano-López et al. 1997). The differences pointed out by the results in the current study proved that in both climatic events, there was real impact on the reproductive stages, suggesting that in the El Niño event, the environmental conditions were most favorable and that this caused intervals among the reproductive stage to be shortened and the gonads to recuperate rapidly, accelerating the ripe stage. Similar results were found by Arntz et al. (1988) and Wolf (1988) during El Niño 1982–1983, in *Argopecten purpuratus* (Pectinidae) at the Peruvian coast, where they observed especially high intensity on spawn.

The sex ratio in both periods did not differ from the expected 1M:1F ratio—this result coincides with those of Saucedo-Lastra and Monteforte (1997) and Solano-López et al. (1997), 1M:0.38F and 1.6M:1F ratios, respectively—but differs from those reported by García-Domínguez et al. (1996) where the females predominated (1.33F:1M). The hermaphroditic frequency in the current study was higher than those reported by García-Domínguez et al. (1996) and Saucedo-Lastra and Monteforte (1997), 0.64% and 0.6%, respectively. In both periods, the highest hermaphroditic frequency was present during the main season of reproductive activity, while males predominated, coinciding with Sevilla (1969) and Saucedo-Lastra and Monteforte (1997) in *P. mazatlanica* and by Behzadi et al. (1997) in *P. fucata*. The results in the current study and others obtained by several authors confirm the protandric condition of *P. mazatlanica* (García-Domínguez et al. 1996, Saucedo-Lastra & Monteforte 1997, Sevilla 1969), as occurs in other *Pinctada* species (Behzadi et al. 1997 and Tranter 1959 in *P. fucata*; Tranter 1958a in *P. albina*; Tranter 1958b in *P. margaritifera*; and Rose et al. 1990 in *P. maxima*).

During the El Niño, the occurrence of highest mean values of oocyte diameter corresponding to ripe stage and the lowest mean values corresponding to the spawn and spent stages allow us to infer that greater oocyte size were liberated to the external environment. This relationship between oocyte diameter and gonadal stages of *P. mazatlanica* reflects the gametogenic cycle, as clearly occurs in other bivalve species studied in normal environmental conditions: *Glycymeris gigantea* (Villalejo-Fuerte et al. 1995), *Spondylus leucacanthus* (Villalejo-Fuerte & García-Domínguez 1998), and *Periglypta multicostata* (García-Domínguez et al. 1998). This relationship did not occur in the La Niña event.

The maximum and minimum CI values and their fluctuation tendency, in both periods, were similar. This relationship between CIs to the reproductive process suggests a strong influence of the gonad weight over the general organism condition, as was reported for *P. mazatlanica* by Solano-López et al. (1997) in the Gulf of Nicoya, Costa Rica, and also reported for another bivalve species by Ochoa-Baez (1985) for *Modiolus capax* and by Villalejo-Fuerte and Ceballos-Vázquez (1996) for *Argopecten circularis*. These CI fluctuations are associated with the nutritional and reproductive condition of mollusks (Searcy-Bernal 1984), and for this reason we can consider CI a general condition indicator to the specimens that conform *P. mazatlanica* reproductive population and also as the general reproductive process.

The *P. mazatlanica* reproductive cycle showed a seasonable relation to SST. The minimum gonadic activity (indifferent stage) occurred during autumn at El Niño and in winter at La Niña; the highest developing frequency during summer at El Niño and in winter at La Niña; and the ripe stage in the close of spring at El Niño and in middle summer at La Niña. In both periods, when the temperature reached 27°C (middle summer at El Niño, close of summer at La Niña), spawning began and was maintained during all the time (months) while the temperatures were about 30°C (late autumn during El Niño and early autumn during La Niña), and spawning in both periods was suspended at temperatures under 25°C. This crucial interval could be considered as optimum for *P. mazatlanica* spawning and is similar to those reported in the same species by Sevilla (1969), Tripp-Quezada (1991), Arizmendi-Castillo (1996), García-Domínguez et al. (1996), and Saucedo-Lastra and Monteforte (1997). The difference in both periods was that the reproductive event in El Niño was longer in time and more intensive on population spawning frequency, and all this explained was happened to *P. mazatlanica* by García-Domínguez et al. (1996) on "El Niño" 1991–1992, at Isla Espíritu Santo, B.C.S., Mexico, where spawning activity occurred throughout the study period (June 1992 to August 1993). In La Niña, the spawning activity was shorter in time length and began until the 27°C temperature was reached (August 1999). For both periods, temperature was inversely related to CI, and CI was closely related to condition of specimens and they, on lowest temperature periods (winter season), were in indifferent and developing stages having the highest condition values. During the highest temperature periods (summer season), spawning was present and hence the minus CI values.

The oocyte mean diameter during El Niño was related to the ripe and spawning stage and CI, all of them influenced by temperature variations, as pointed out above. During La Niña, this relationship did not occur at all.

Pigment concentration in both periods presented similar sea-

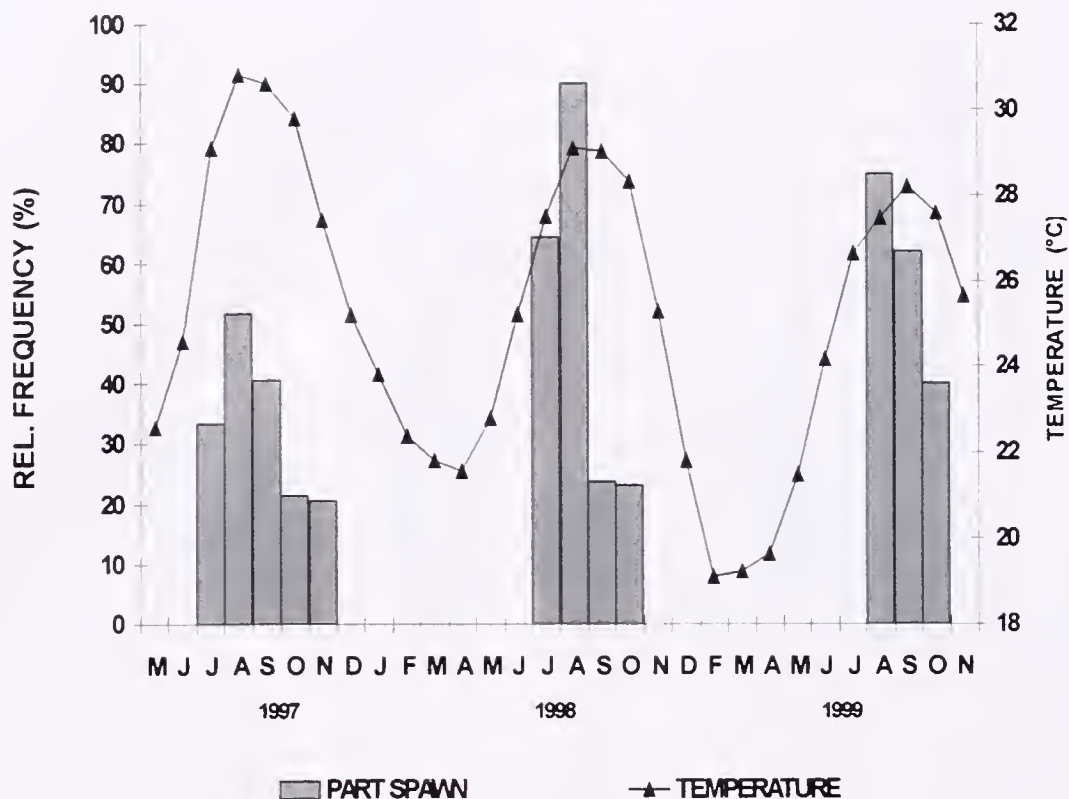


Figure 6. Relationship between the sea surface temperature and the relative frequency of the part-spawning stage from May 1997 to November 1999 at Isla Espiritu Santo, Gulf of California.

sonal fluctuations. The difference between both periods was that during El Niño, the mean, maximum, and minimum pigment concentrations were smaller than those during La Niña. In *P. mazatlanica*, for both periods, gametogenic production (developing

stage) started during winter, coinciding with highest photosynthetic pigment concentration. During ripe and spawning stages, no relationship was found in both periods with pigment concentration, and we assume that these events were realized at the expense of

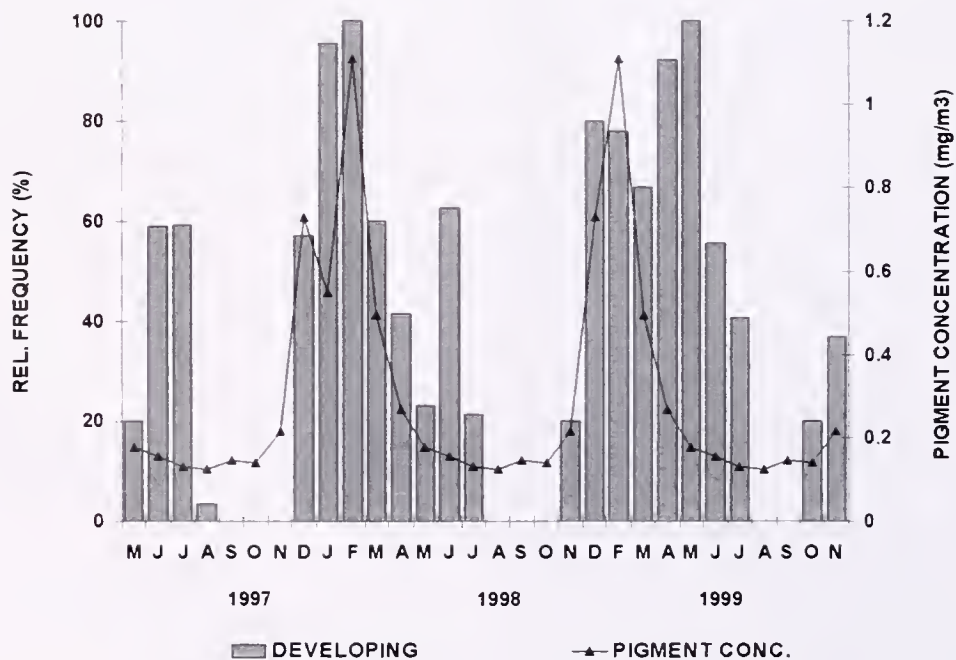


Figure 7. Relationship between the photosynthetic pigment concentration and the relative frequency of the developing stage from May 1997 to November 1999 at Isla Espiritu Santo, Gulf of California.

organism nutrient reserves. There are observations in many bivalves that gametogenic process takes place at the expense of conjunctive reserve tissue (Galtsoff 1964, Mathieu and Lubet 1993), as the case in the mussel *Perumytilus purpuratus* (Lozada and Reyes 1981) during spawning and in *P. mazatlanica* (García-Gasca 1992) during gamete formation.

During El Niño, pigment concentration was related to CI, but not during La Niña, in this case the fluctuations of CI could be attributed to the irregular gonadic process, as occurred during the developing and ripe stages presented by the specimens during this period. The monthly mean oocyte diameter, in both periods, was not related to pigment concentration, as the maximum monthly mean diameters occurred in summer.

Finally, it may be concluded that temperature was the main factor regulating the spawning time during the El Niño and the La

Niña, and that food availability was not related to the pearl oyster spawning.

ACKNOWLEDGMENTS

This study was conducted as part of an Institutional Program of the Centro Interdisciplinario de Ciencias Marinas (CICIMAR-IPN, Mexico), which is funded by Consejo Nacional de Ciencia y Tecnología (CONACYT-Mexico) and Consejo Nacional de Educación Tecnológica (COSNET-México). Our gratitude to Manuel Zamarrón and Ciro Arista for their help in collecting samples, Fidel Camacho and Jorge López for their work on histologic processing, and the CIBNOR editorial staff for review of the English text. Daniel Lluch-Belda and Federico García-Domínguez received a grant from the Comisión de Operación y Fomento de Actividades Académicas of the Instituto Politécnico Nacional.

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RESPONSE PROFILE OF THE CALAFIA PEARL OYSTER, *PINCTADA MAZATLANICA* (HANLEY, 1856), TO VARIOUS SEDATIVE THERAPIES RELATED TO SURGERY FOR ROUND PEARL INDUCTION

MARIO MONTEFORTE,* HORACIO BERVERA AND PEDRO SAUCEDO

Centro de Investigaciones Biológicas del Noroeste, S.C. (CIBNOR), La Paz,
Baja California Sur, CP 23090, México

ABSTRACT Anesthetic drugs are common in management sequences of commercial aquaculture that imply relaxation of specimens. Studies on physiologic response and relationships with functions of body parts are particularly centered in fishes to improve transport, handling, or surgery (i.e., spawning stimulation, sampling of blood and fluids). In pearl oysters, relaxation therapies are important to induce round pearl formation with nucleus-mantle insertions, but the physiologic effect of drugs are poorly known. In the current work, respiration rate (R_r , in $\mu\text{L L}^{-1} \text{g}^{-1} \text{min}^{-1}$) was used as index to establish recovery profiles in *Pinctada mazatlanica* and evaluate relaxation therapies of three doses and two exposure times using propylene phenoxetol (PP), MS-222 (MS), and quinaldine (QN), in comparison with an untreated (CTRL) model. Recovery profiles were explored with median Box-Whiskers plots. Intercept position, dispersion pattern, and range values of R_r and slope attributes, were inferred through correlation and regression methods. Relationships among these elements were analyzed with three-way ANOVA (time \times dose \times exposure time); recovery profiles were compared within and between drugs through multiple covariance and cluster analysis to explain physiologic responses. Two-way ANOVA at start and end of each trial provided further elements for therapy evaluation. Specimens responded dynamically in this experiment, sharing an overall background variation of R_r strongly influenced by time but with wide differences of significance P level, between 10^{-18} in CTRL and 10^{-10} to $<10^{-24}$ between drugs. CTRL, MS, and PP had negative correlation of R_r over time, whereas it was positive for QN. The influence of dose had grouped P levels between 10^{-11} to 10^{-15} ; exposure time and interactions with dose shared P levels of similar significance between 10^{-3} to 10^{-5} . In comparison with the CTRL model, the use of drugs apparently contributed to decreased stress response in treated specimens, and this was particularly notable in early recovery phases. Variations in dose and exposure time defined specific recovery profiles within drugs. An adequate presurgery therapy should sustain low intercept, low dispersion and range value of R_r , and horizontal slope. QN at doses of 45 mL and PP at 5 mL propiciated low-activity profile during recovery.

KEY WORDS: MS-222, *Pinctada mazatlanica*, propylene phenoxetol, quinaldine, relaxation therapy

INTRODUCTION

Surgery techniques for round pearl formation in pearl oysters have been examined since the early 1940s in publications, reports, manuals, congresses, reunions, and training courses (Gervis 1992, Fassler 1994, SPC-POIB). Videotapes, photography, and the World Wide Web have also become important sources of information. The general agreement recognizes that a pearl culture technician may qualify as "trained" after thousands of essays; even then, differences of success rate (nucleus retention, survival, number and quality of pearls) are notable among technicians and species (Alagarwami 1987, Shirai 1994, Monteforte 1996, Haws 1998, Haws et al. 1999, Nava et al. 2000). Many inherent factors have their own influence, such as the biologic state and health of pearl oysters, response profile to presurgical and healing therapies, quality and profilaxis of workspace, and so forth. Field culture management and environmental conditions also play an important role. Pearl culture technicians coincide in two major requirements that specimens should fulfill before the surgery: (1) newly spawned gonads, empty but prominent enough for assuring nucleus and mantle piece insertion, and (2) adequate relaxation, valves can be easily gapped and body contraction will be slow during the intervention. Thermal shocks and other strategies are applied for spawning induction: anesthetic compounds are commonly used as relaxation treatments (Alagarwami 1987, Ehteshami 1993, Monteforte et al. 1994, Mills et al. 1997, Norton et al. 2000). We investigated the recovery profiles of the Calafia pearl oyster, *Pinctada mazatlanica* (Hanley, 1856), treated with

propylene phenoxetol, MS-222, and quinaldine in different doses and exposure times. *P. mazatlanica* needs adequate presurgery therapies for round pearl stimulation because specimens react defensively if certain level of relaxation is not reached. On the other hand, norms for evaluation of response profiles in mollusks under anesthetics, including pearl oysters (Norton et al. 2000), have been related mainly with behavior (reply level of body parts as a result of mechanical stimulations, time for specimens to attain visible signs of "normal" state, and results in nucleus-mantle retention, survival after cases of expulsion, and total failures). We used the respiration rate as index to describe recovery profiles of the presurgery therapies tested in this study.

MATERIALS AND METHODS

Selection of Therapies

Previous essays with menthol and benzocaine gave deficient results in presurgery sedation of *P. mazatlanica*. Propylene phenoxetol was tested in *P. maxima* (Mills et al. 1997) and *P. margaritifera* (Norton et al. 2000). Both species had suitable relaxation with 2.5 to 5 mL L^{-1} at exposures of 15 to 20 min, but total failures in round pearl induction in *P. margaritifera* were associated with this drug (Norton et al. 2000). Heasman et al. (1995) found no apparent effect in *Pecten fumatus* at doses of 0.6 mL L^{-1} . In *P. radiata*, Ehteshami (1993) obtained good results with 1 mg L^{-1} of MS-222. The effect of this substance has been investigated in a wide range of doses and exposure times for several species of fishes and mollusks (i.e., *Oncorhynchus mykiss* by Wagner et al. 2002; *Ostrea edulis* by Culloty & Mulcahy 1992; *Haliotis iris* by Aquilina and Roberts 2000; *Elliptio complanata* by Lellis & Plerhoples 1997; and Lellis et al. 2000). Heasman et al.

*Corresponding author. Fax: (+612) 125-3625; E-mail: montefor04@cibnor.mx

(1995) reported hyperactivity and hiperextension of tentacles in *Pecten fumatus* when exposed to 0.1 g L^{-1} of MS-222. Quinaldine is commonly used in fish transport and broodstock handling; as in the previous drugs, there are different exposure times or doses reported as efficient, depending on the species and type of studies (i.e., *Sparus aurata*, Kumlu & Yanar, 1999; Massee et al. 1995 in larvae of *Sciaenops ocellatus* and *Carassius auratus*). Studies on the effect of quinaldine in mollusks are rare. Heasman et al. (1995) observed valve closure in *Pecten fumatus* when exposed to this substance. Apparently, there is no information regarding pearl oysters.

Experimental Design and Data Treatment

Approximately 3300 cultivated *P. mazatlanica* (wild spat collection) 14 to 16 mo old, were selected for this experiment in September 2001. We confirmed 39/45 specimens in rest or post-spawning stage as expected in the end of reproduction season (Saucedo & Monteforte 1997). A total of 342 were size-selected and divided in three groups of 108 individuals corresponding to each drug: propylene phenoxetol (PP), MS-222 (MS), and quinaldine (QN), plus 18 as controls (CTRL). CTRL monitoring was made in the first place to establish the "standard" profile, then PP, MS, and QN. In each session, pearl oysters were cleaned, measured for shell height (mean = $108.8 \pm 6.7 \text{ mm}$ SD), and left in starvation for 24 h in a 1500 L tank with filter-aerated seawater stable at $24 \pm 1.5^\circ\text{C}$ SD throughout the experiments, which is optimal for the species (Saucedo et al. 2003). With exception of CTRL, trials of 18 individuals were exposed to 3 different doses for each drug during 30 and 60 min into 20 L containers filled with filtered seawater (Table 1). These variables were deduced from the therapies previously outlined. When concluded, a particular exposure time, wet weight (mean = $161.2 \pm 33.1 \text{ g}$ SD) was measured after placing pearl oysters in ventral position and open for few seconds to drip water excess. Along with 9 untreated individuals per "exposure" time, they were transferred into respective respiration chambers already stabilized on oxygen concentration (Saucedo et al. 2003). Three YSI-58 oxygen-meters provided records every 10 or 20 min (Table 1), ending when the variation between subsequent measurements trended to stabilize and/or to appear alike CTRL. Calibration of YSI meters was checked at start and end of each experimental session in comparison with blank

respirations chambers (1 per session). Normality for shell height was confirmed with a K-S test ($d = 0.10$, $P > 0.2$). Wet weight had differences, but the significance level was conservative ($d = 0.15$, $*P < 0.05$). Correlation of both dimensions was positive and significant ($F = 278.75$, $R = 0.87$, $***P < 0.001$). Saucedo and Monteforte (1997) and Saucedo et al. (1998) considered wet weight acceptable as identification tag for individuals this size/age. In our study, similar overall reproductive stage among individuals would have contributed to reduce wet weight outliers. Respiration rate (Rr) was analyzed as index of recovery within and between drugs, and related to CTRL, through the formula:

$$\text{Rr}(\mu\text{L L}^{-1} \text{ O}_2 \text{ g}^{-1} \text{ min}^{-1}) = [(\text{OxTo} - \text{OxTn})/\text{Wet weight}]/\text{Tn}$$

OxTo and OxTn are starting and subsequent YSI records in mg L^{-1} of oxygen, converted to $\mu\text{L L}^{-1}$ of O_2 (Lehninger 1982). This unit was convenient for the strategies of data management and definition of plot patterns in which the purposes of this work were based. Wet weight (in g) is the individual tag, and Tn is the corresponding recovery time stage expressed in logs (T) of 10 or 20 min. Response profile was explored with median Box-Whiskers tests on raw Rr data (Fig. 1), this underlines individual behavior and its influence on profiles which represents important criteria for therapy evaluation. Slope attributes and intercept of data sets were obtained by regression methods. Their reliability was inferred with one-way ANOVA F and corresponding regression coefficient (R). Covariance analyses were calculated to assess differences in slopes ($F_{\text{calc}} > F_{\text{tab}}$, $*P < 0.05$) or elevations ($F_{\text{calc}} < F_{\text{tab}}$, $P > 0.05$) (Table 2). The influence of recovery time on effects of dose, exposure time, and their interactions, was examined with three-way ANOVA (time \times dose \times exposure) and compared with CTRL based on their respective P level of significance (Table 3). To recognize main effects of dose and exposure time within drug, the post-hoc HSD Tukey sequence was applied with recovery time as contrast background. Further elements for therapy evaluation were provided by two-way ANOVA and HSD Tukey on Rr records at the start and end of each experiment (Table 4) (Sokal and Rohlf 1981, Dillon and Goldstein 1984, Zar 1999, CSS-Statistica 1999). Survival was monitored 6 mo in field culture installations.

RESULTS

Blank respiration chambers remained stable in all sessions between 6.55 and 6.70 mg L^{-1} . Calibration of YSI meters was seldom needed. Untreated (CTRL) *P. mazatlanica* presented highly significant negative correlation of Rr over time ($F = 121.2$, $R = 0.85$, $***P < 0.001$). Intercept was $0.20 \mu\text{L L}^{-1} \text{ g}^{-1} \text{ min}^{-1}$ and slope -0.004 (Table 2); the P level of "recovery" time layed on 10^{-18} (Table 3). Overlap of time-logs in CTRL occurred between T40 and T50 (Fig. 1). Final record of Rr at T50 was $0.05 \pm 0.01 \mu\text{L L}^{-1} \text{ g}^{-1} \text{ min}^{-1}$ (Table 4). Some individuals at T30 also had low Rr, whereas T10 was grouped highest (Fig. 1). Significant negative correlation was also detected for pearl oysters treated with PP and MS ($F = 28.39$ and 177.62 , $***P < 0.01$), whereas for QN the correlation was positive ($F = 114.69$, $***P < 0.01$). The general response profile in all drugs was highly dependent on recovery time with P levels of 10^{-10} to $< 10^{-24}$; particular patterns were noted among treatments and in comparison with CTRL (Fig. 1, Tables 2–4). Trials into PP and MS seemed to share a common model near to CTRL, whereas notable differences were detected in pearl oysters exposed to QN (Fig. 1). R coefficient was low in PP and better in MS (0.37 and 0.74, respectively), whereas in QN was 0.61; CTRL had 0.85. This review indicated intrinsic effects of

TABLE 1.

Experimental design of anesthetic therapies with three drugs.

Drug Dose	Exposure Time	Recovery Time
	(min)	(min)
Propylene phenoxetol (PP) 1 mL, 3 mL, 5 mL	30	10
		20
		40
Finquel MS-222 (MS) 2.5 mg, 5 mg, 7.5 mg	60	30
		40
		50
Quinaldine (QN) 15 mL, 30 mL, 45 mL	Untreated	60
		80
		10 to 50
Control (CTRL) ($n = 18$)	$(n = 9 \text{ sims/Exp. T.})$	

Trials under treatment (dose, exposure time) consist of $n = 18$ individuals each. CTRL specimens were divided in groups of $n = 9$ for each "exposure" time. Experimental sessions were not simultaneous: CTRL was first, PP second, MS third, and QN last.

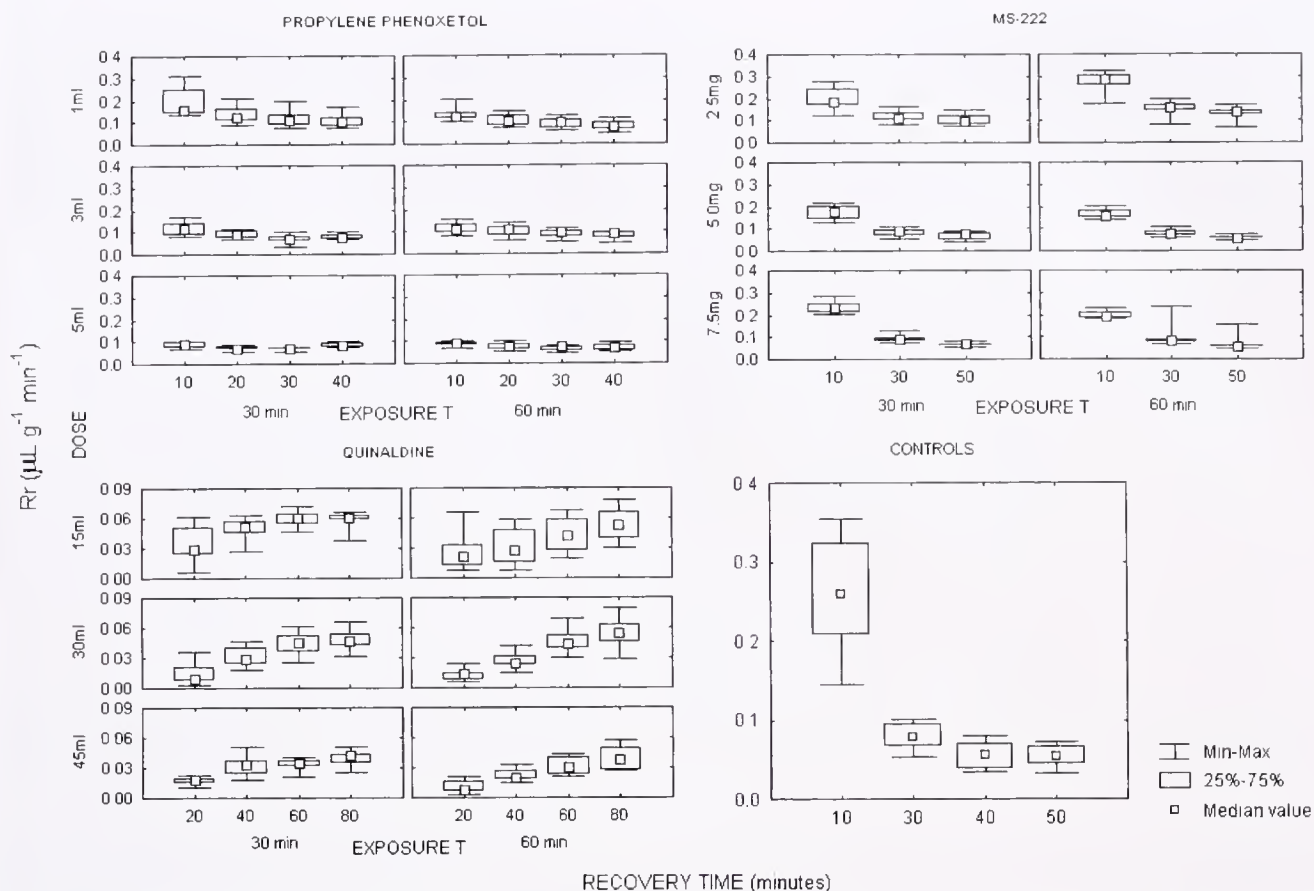


Figure 1. Median Box-Whiskers plot of recovery profiles measured by Respiration rate in *Pinctada mazatlanica* under three relaxation therapies (PP, MS, and QN) in comparison with a model (CTRL).

drug in the recovery profiles of *P. mazatlanica*. Dose and/or exposure time delineated particular patterns within and between drugs. Results in CTRL were essential to understand these profiles. We examined each experiment.

Propylene Phenoxytol

Significant negative correlation of Rr related to recovery time was confirmed for most cases ($**P < 0.01$). In these, Rr intercept was from 0.12 to 0.21 $\mu\text{L L}^{-1} \text{g}^{-1} \text{min}^{-1}$, R settled into 0.52 and 0.64, and slope into -0.003 and -0.001 . Individuals at dose of 5 mL in both exposure times were exceptions (Fig. 1, Table 2). The regression test and R values seemed sensible to individual differences within PP, particularly in these exceptions. However, the overall Rr range was lower than in MS or CTRL (Fig. 1, Tables 2 and 4) and tendency to horizontality seemed more notable (Fig. 1, Table 2). The covariance analysis indicated highly significant differences in slopes ($F_{\text{calc}} = 4.40 > F_{\text{tab}} \approx 2.27$, $***P < 0.001$) and elevations ($F_{\text{total}} = 33.55 > F_{\text{tab}} \approx 2.81$, $***P < 0.001$). The effect of dose had important influence in these estimates, recovery time shortly followed as evident background, whereas exposure time had less significance; P level of dose and exposure time was similar as in other drugs (Table 3). Interaction of dose with exposure time, and of recovery time with dose also had rather equivalent significance as in MS and QN, while nonsignificant influence of recovery time was detected on the effect of exposure time or the combination of all effects (Table 3). Dose of 5 mL at both exposure times tended to horizontality from T10. Recovery time had no

influence as all-effect, confirming a common background response profile for all PP treatments without major Rr outliers (Fig. 1, Table 3). The three-way post-hoc HSD Tukey test with contrasted recovery time indicated that pearl oysters had low Rr in high dose or long exposure. On the contrary, low dose and short exposure introduced data dispersion, high intercept value and more slope inclination (Fig. 1, Table 2). Exposure for 60 min promoted homogeneity of recovery profiles for all doses; the general Rr range in each time stage tended to be low and narrow (Fig. 1, Tables 2 and 4). Pearl oysters exposed for 30 or 60 min into 5 mL had stable Rr during recovery, with nearly horizontal slope and low intercept value (Fig. 1, Tables 2 and 4). The two-way ANOVA-HSD Tukey at T10 of recovery indicated highly significant differences of Rr values for dose ($F = 15.22$, $***P < 0.001$) but not for exposure time ($F = 3.54$, $P > 0.05$), neither their interaction ($F = 2.78$, $P > 0.05$). In this stage, individuals treated with higher dose and/or longer exposure time had lower and narrower Rr, coinciding with low intercept and almost horizontal slope (Fig. 1, Table 4). At T40 there was no effect of dose ($F = 2.8$, $P > 0.05$), whereas exposure time had moderated importance ($F = 5.23$, $*P < 0.05$). Their interaction was not significant ($F = 2.0$, $P > 0.05$). In this stage, differences of Rr values were significant only between batch of 1 mL–30 min (highest Rr) and the one of 5 mL–60 min (lowest Rr) (Fig. 1, Table 4). Survival was 100%.

MS-222

Correlation of Rr over time was negative and significant in all treatments ($***P < 0.001$). The intercept varied between 0.18 and

TABLE 2.

Statistical calculations to infer regression attributes and covariance comparisons in recovery profiles of the Catalina pearl oyster, *Pinctada mazatlanica*, under different relaxation therapies with propylene phenoxetol (PP), MS-222 (MS) and quinaldine (QN).

Group	Exposure	Dose	Rr Intercept ($\mu\text{L g}^{-1} \text{min}^{-1}$)	Slope	ANOVA F Regression	R. Regression Coefficient	Covariance	Post hoc HSD Tukey
CTRL	—	—	0.20	-0.004	121.22***	0.85	-0.66	—
PP	30	1 mL	0.21	-0.003	15.05***	0.64	-0.26	d
		3 mL	0.12	-0.001	14.99***	0.64	-0.17	abc
		5 mL	0.08	-0.000	0.32	0.12	-0.06	ab
	60	1 mL	0.15	-0.002	11.93***	0.59	-0.23	c
		3 mL	0.13	-0.001	8.23**	0.52	-0.14	bc
		5 mL	0.09	-0.001	4.17	0.40	-0.04	a
MS	30	2.5 mg	0.21	-0.002	22.66***	0.71	-0.67	c
		5.0 mg	0.19	-0.003	48.72***	0.83	-0.75	ab
		7.5 mg	0.26	-0.004	92.55***	0.90	-1.17	bc
	60	2.5 mg	0.30	-0.004	45.28***	0.82	-1.03	d
		5.0 mg	0.18	-0.003	83.86***	0.89	-0.74	a
		7.5 mg	0.22	-0.003	37.01***	0.79	-0.91	abc
QN	30	15 mL	0.03	0.0004	11.65**	0.56	0.17	c
		30 mL	0.01	0.0006	36.68***	0.76	0.21	ab
		45 mL	0.01	0.0003	22.55***	0.68	0.11	ab
	60	15 mL	0.01	0.0005	8.60**	0.50	0.12	b
		30 mL	-0.001	0.0007	91.82***	0.88	0.23	ab
		45 mL	0.002	0.0004	47.42***	0.80	0.22	a

Significance of the regression ANOVA F is * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The post hoc HSD Tukey considered recovery time as contrast factor. Different indexes denote significant differences at * $P < 0.05$.

0.30 $\mu\text{L L}^{-1} \text{g}^{-1} \text{min}^{-1}$ and R between 0.71 and 0.90. Slope values predominated in -0.003 and -0.004, with exception of one only case of -0.002 (Fig. 1, Table 2). The overall range of R coefficient appeals for more uniform variation of Rr within MS, frequently over 0.79 (Table 2), but this may not be clinically conclusive. Covariance analysis revealed conservative differences in slopes ($F_{\text{calc}} = 2.32 > F_{\text{tab}} \approx 2.27$, * $P < 0.05$) while elevations appeared more diverse ($F_{\text{total}} = 28.31 > F_{\text{tab}} \approx 2.81$, *** $P < 0.001$) (Fig. 1, Table 2). The effect of time in the recovery profiles under MS seemed most important than in other drugs (Table 3). Dose, exposure time, and their interaction were significant with similar P level as in PP and QN. All-effect interaction was significant but at lowest P level; although code mixing was frequent at T50, overlap order of T30 and T50 occurred seldom within sets and most cases

of T10 tended to group at higher Rr (Fig. 1). This suggests that the influence of recovery time was probably overestimated: the inclusion of a T40 log would have increased overlapping events between this and T50 (as in PP and CTRL), while the inclusion of a T20 would have scarcely affected the first (higher Rr) position of

TABLE 4.

Results of two-way ANOVA and HSD Tukey at first and final Rr for each drug therapy.

Group	Exposure (min)	Dose	ANOVA F First Rr (Mean \pm SD)	ANOVA F Final Rr (Mean \pm SD)
CTRL	—	—	0.25 \pm 0.07	0.05 \pm 0.01
PP	30	1 mL	0.19 \pm 0.09 b	0.11 \pm 0.03 b
		3 mL	0.12 \pm 0.03 a	0.08 \pm 0.01 ab
		5 mL	0.09 \pm 0.01 a	0.08 \pm 0.01 ab
	60	1 mL	0.13 \pm 0.07 ab	0.08 \pm 0.02 ab
		3 mL	0.12 \pm 0.03 a	0.08 \pm 0.02 ab
		5 mL	0.09 \pm 0.01 a	0.07 \pm 0.02 a
MS	30	2.5 g	0.20 \pm 0.05 ab	0.10 \pm 0.03 hc
		5.0 g	0.17 \pm 0.04 a	0.07 \pm 0.02 a
		7.5 g	0.23 \pm 0.03 bc	0.07 \pm 0.01 a
	60	2.5 g	0.28 \pm 0.05 c	0.13 \pm 0.03 c
		5.0 g	0.17 \pm 0.02 a	0.06 \pm 0.01 a
		7.5 g	0.20 \pm 0.02 ab	0.07 \pm 0.04 ab
QN	30	15 mL	0.035 \pm 0.019 b	0.058 \pm 0.009 b
		30 mL	0.014 \pm 0.011 a	0.048 \pm 0.010 ab
		45 mL	0.017 \pm 0.004 ab	0.040 \pm 0.008 a
	60	15 mL	0.026 \pm 0.019 ab	0.053 \pm 0.017 ab
		30 mL	0.013 \pm 0.006 a	0.054 \pm 0.015 ab
		45 mL	0.011 \pm 0.007 a	0.040 \pm 0.011 a

CTRL, control; PP, propylene phenoxetol; MS, MS-222; QN, quinaldine.

TABLE 3.

Results of three-way ANOVA to determine influence and P levels of factors and interactions in the recovery profiles of treated and untreated *Pinctada mazatlanica*.

Effect	CTRL	PP	MS	QN
Recovery time	2.20×10^{-18}	2.11×10^{-10}	$<10^{-24}$	1.99×10^{-24}
Dose	—	2.97×10^{-14}	4.56×10^{-15}	4.91×10^{-11}
Exposure time	—	1.24×10^{-2}	4.84×10^{-2}	1.84×10^{-3}
Recovery time vs. dose	—	7.81×10^{-3}	0.86	0.22
Recovery time vs. exposure time	—	0.34	0.91	0.15
Dose vs. exposure time	—	1.63×10^{-4}	1.52×10^{-5}	9.75×10^{-3}
All effects	—	0.81	2.76×10^{-2}	0.96

CTRL, control; PP, propylene phenoxetol; MS, MS-222; QN, quinaldine.

T10 logs (i.e., T10 of datasets 2.5 mg–60 min and 7.5 mg–30 min). The sudden rise of Rr at T30 and T50 in trial of 7.5 mg–60 min corresponds to three specimens (Fig. 1), apparently provoked by mechanical disturbances (noise or motion). The contrasted three-way HSD Tukey indicated that pearl oysters had high Rr and dispersion at lower doses for both exposure times. The experiment of 2.5 mg–60 min presented highest slope and intercept value with dispersion toward high Rr. Individuals placed into 5 mg for 30 and 60 min had less variation in their recovery profile, also coinciding with lowest intercept and “average” -0.003 slope. Longer exposure time in doses of 5.0 and 7.5 mg apparently contributed to decrease individual differences; however, this effect was only observed when comparing early recovery stages of both exposure times (Fig. 1, Tables 2 and 4). A related analysis with elimination of outliers in T30 and T50 for 7.5 mg–60 min dataset did not alter the similarity of trials with 5.0 mg for both exposure times. The two-way ANOVA-HSD Tukey test applied at T10 and T50 of recovery supports the above. In the first time-log, the effect of dose was highly significant ($F = 14.95$, $***P < 0.001$), so was its interaction with exposure time ($F = 11.19$, $***P < 0.001$), while this last apparently had no influence ($F = 1.19$, $P > 0.05$); in the second time-log, only the effect of dose was significant ($F = 23.97$, $***P < 0.001$). At T10, individuals of 7.5 mg–30 min and 2.5 mg–60 min had significantly higher Rr (also higher intercept and slope inclination) than those treated with 5 mg at both exposure times (lower intercept and “average” slope) (Tables 2 and 4). At T50, pearl oysters placed into 2.5 mg during 30 and 60 min of exposure had higher Rr, while the rest were relatively similar (Table 4). Elimination of outliers 7.5 mg–60 min at T50 made no impact in this pattern. Survival was 100%.

Quinaldine

All treatments had significant positive correlation of Rr over time ($***P < 0.01$). R coefficient varied between 0.50 and 0.88, intercept from 0.001 and $0.03 \mu\text{L L}^{-1} \text{g}^{-1} \text{min}^{-1}$, and slope from 0.0003 to 0.0007 (Table 2). Overall range of R values suggests slightly better reliability of this predictors than in PP. Besides, the absolute variation of Rr within QN was narrow and had lower values than in other drugs and CTRL, almost throughout the experiment (Fig. 1). The covariance analysis indicated similarity of slopes ($F_{\text{calc}} = 1.84 < F_{\text{tab}} \approx 2.27$, $P > 0.1 < 0.25$), whereas elevations were highly different ($F_{\text{total}} = 58.54 > F_{\text{tab}} \approx 2.81$, $***P < 0.001$). The effect of recovery time was very important in this pattern at P level of 10^{-24} . Similar to other drugs and CTRL, Rr of pearl oysters under QN followed a main background profile related with time, although in positive direction. Dose also had strong influence but at relatively less extent than in PP or MS, whereas exposure time and its interaction with the former had similar P level as in other drugs. All-effects interaction was not significant (Table 3). Overlap of time-logs occurred in all datasets for T60 and T80 min of recovery (Fig. 1), but in some trials this started earlier coinciding with larger individual differences of Rr (i.e., from T20 in 15 mL–60 min, and from T40 in 45 mL–30 min). Cases of T20 tended to group in last place (low Rr) except those of 15 mL (Fig. 1). The three-way HSD Tukey with recovery time as contrast suggests significant differences between lowest Rr in 45 mL–60 min and highest in 15 mL–30 min, coinciding respectively with low and high intercept and similar slope. Differences of recovery profiles in treatments with 30 and 45 mL at both exposure

times were not substantial. Response homogeneity among individuals seemed to increase with dose, while variation of Rr increased with longer exposure times. R value of each batch also indicate individual differences in 15 mL at both exposure times, while moderate to low dispersion was detected for other treatments (Table 2). However, the general trace of Rr values over recovery time did not tend to dispersion declining as in other drugs or CTRL (Fig. 1). In some treatments, individual differences remained rather constant, either in low Rr such as those of 45 mL in both exposure times or high Rr like 15 mL in both exposure times. Dispersion increase seemed more evident in dose of 30 mL at both exposure times (Fig. 1, Table 2). The analysis at T20 and T80 of recovery stands for this pattern (Table 4). At T20, the effect of dose was significant ($F = 9.12$, $***P < 0.001$), while not the effect of exposure time ($F = 2.39$, $P > 0.1$), neither their interaction ($F = 0.37$, $P > 0.5$). Batches of 15 mL–30 min and 15 mL–60 min had higher Rr but dispersion weighed toward low values in the first and high values in the second (Fig. 1); difference between these was not significant. Significantly low Rr oscillated between 0.011 and $0.014 \mu\text{L g}^{-1} \text{min}^{-1}$ at T20, the first—and lowest of all drug sets—corresponding to 45 mL–60 min. At T80, the effect of dose was significant ($F = 6.44$, $**P < 0.01$), whereas exposure time had no effect ($F = 0.003$, $P > 0.5$). Their interaction was not significant ($F = 0.82$, $P > 0.1$). Differences of Rr were evident only between 15 and 45 mL in the same exposure time (30 min). The first started (T20) and finished (T80) with significantly higher Rr; it also presented the highest intercept value and similar slopes for each exposure time but also the lowest R value. The second had lower Rr, coinciding with low intercept and the most horizontal slope of each exposure time; R values were higher than in doses of 15 mL. Apparently, individuals treated with 45 mL still had low Rr at T80 (Fig. 1, Tables 2 and 4). There was no mortality during the experiment.

Mortality in Field Culture

The generation of *P. mazatlanica* corresponding to this study accumulated 4% of average mortality in six months, with frequent monthly zeroes in many containers. This behavior is normal for the species (Monteforte et al. 2000). In general, the current experiment did not diverge from this pattern. Mortality after 6 mo was: 1 individual for CTRL and QN, respectively, and 2 for PP and MS, respectively. We noted that all incidence of mortality occurred in the first month of field culture. Survival was 100% until the sixth month.

DISCUSSION

Evaluation of presurgery therapies for *P. mazatlanica* was based on the effect of dose, exposure time, and interactions within and between drugs, and their comparison with the model pattern of CTRL. These therapies were explained as index of recovery or “stress level” by the position of intercept and Rr value at starting T, by the pattern of dispersion and range of individual differences during recovery, and by the attributes of slope, including time needed for apparent stabilization toward a “common” Rr value (Fig. 1, Tables 2 and 4). Stress has been interpreted as physiologic response involving particular meaning when related to anesthetics. Records of corticosteroids have been used in studies regarding stress indexes for several species of fish. In most cases, drugs themselves appeared as stressors and did not reveal special advan-

tages to prevent physiologic stress responses (Wagner et al. 2002). In our work, the monitoring of Rr was considered a reliable index to interpret the response of *P. mazatlanica* under different therapies. The model pattern in CTRL started at initial T with differential activity, most individuals into conditions of high intercept and Rr value, followed by rapid decrease of dispersion and Rr value until apparent stabilization at about 0.05 mL L^{-1} in T50 (Fig. 1, Tables 2 and 4). Significance of recovery time in this model was on 10^{-18} P level (Table 3). Pearl oysters under therapy also were strongly influenced by recovery time but with wide differences of P level from 10^{-10} to $< 10^{-24}$ between drugs (Fig. 1, Table 3). This suggest that *P. mazatlanica* had a dynamic overall response during recovery acting as main background variation and strongly affected by time, while dose, exposure time, and their interactions contributed to arrange particular patterns for each therapy regarding the CTRL model. Dose had equivalent P level in PP and MS, and relatively less for QN; the effect of exposure time and interaction with dose were also grouped (Table 3). Differences in significance P level of recovery time may reside on the nature of drug components and their specific influence on mechanical and physiologic functions of also specific body parts (Iversen et al. 2003). In these terms, the appraisal of relationships among treatments and therapy profiles was supported with a cluster analysis of the multiple covariance matrix using complete linkage rule and Euclidian distances (Sokal and Rohlf 1981, Dillon & Goldstein 1984, Zar 1999, CSS-Statistica 1999). As expected, the effect of drug appeared as important discriminant between treated and untreated individuals (Fig. 2). Main clusters distinguished recovery profiles based on activity level, from higher in CTRL to lower in QN (Fig. 1, Tables 2 and 4). CTRL stated at 60% of definition. Trials into PP, MS seemed to share a common model near to CTRL: high activity and/or dispersion of Rr in early phase, dispersion declining toward low Rr as recovery progressed, and apparent stabilization at about 50 min. Aside their different slope attributes, pearl oysters exposed to QN had no evident dispersion declining of Rr through time as in the previous drugs and CTRL; however, overall absolute variation remained narrow and values were much lower in QN. Stabilization was considered present at 80 min (Fig. 1). The "stress" cluster was formed by all MS datasets and PP 1 mL–30 min and it remained to 44%. Pearl oysters in these trials presented lower Rr dispersion than CTRL and also a tendency to decrease over time, however, their recovery profile appeared similar in sev-

eral elements that suggest unadequate relaxation (Fig. 1, Tables 2 and 4). Notably, trial of PP 1 mL–30 min joined this cluster at 16% apparently because it had the highest intercept, slope, and covariance values within this drug. Its attachment to "MS" cluster was on trial MS 2.5 mg–30 min, as confirmed by their similar profiles (Figs. 1 and 2, Tables 2 and 4). Within "MS," clusters were apparently defined by the dispersion pattern of Rr and its position in the plot (intercept and Rr value at initial T). The cluster formed by trials with 5 mL at both exposure times and trial 7.5 mg–60 min indicate homogeneity of Rr toward low-activity profiles (i.e., low intercept, moderate slope, dispersion decrease, and low Rr value at final T) (Fig. 1, Tables 2 and 4). Components of cluster "PP" suggest that higher dose promoted low disturbances of Rr. Treatments of 5 mL in both exposure times joined at 4% and remained as separate cluster up to 20%. Lower dose of this substance and/or lesser exposure time provoked a similar response pattern as in the "stress" cluster, this was notable in the mentioned trial 1 mL–30 min of PP (Figs. 1 and 2; Tables 2 and 4). With this exception, both trials of 3 mL and the one of 1 mL–60 min stayed as cluster of higher activity within PP to 12% (Fig. 2). Cluster "PP" joined the "QN" nearly at 40% with the trials of low-profile PP 5 mL of both exposure times and stayed separated from the previous "CTRL" + "MS" through 100%, however, cluster "QN" remained defined only at 10%. The recovery profile of pearl oysters treated with QN had very low intercept position and Rr value at starting T, and lower range during recovery in comparison with the other experiments. Slope was nearly horizontal but stabilization was considered present later than in CTRL and other treatments. Components of cluster "QN" were apparently defined by the dispersion pattern of Rr and intercept. Low-profile response (lowest of all trials) could be considered for clusters of 45 mL, where dispersion of Rr during recovery was narrow but it remained rather constant. The QN trial of 15 mL–60 min was apparently attached to the former for its tendency to low intercept and constant dispersion, although this last was larger than in doses of 45 mL. Lower dose or exposure time under QN promoted tendencies to increase Rr dispersion; these trials compose the high-activity area within cluster "QN," although their range value remained lower than in CTRL, MS, and PP (Figs. 1 and 2). The overview suggests that resemblance or difference of recovery profiles in CTRL pearl oysters and those under treatment were principally defined during early phases. Trials with MS and PP coincided in having a negative correlation over time indicating recovery from stressed state, but relatively less than in CTRL. Specimens placed into QN had slow recovery from depressed activity toward higher Rr (Fig. 1). This suggest that drugs were no stressors for *P. mazatlanica*. It was also evident that the overall projection of recovery profiles moves toward a Rr of $0.05 \mu\text{L g}^{-1} \text{ min}^{-1}$, assumed from measures in controls at T50 (Fig. 1, Table 4). Specimens under PP and MS had rather similar end Rr as in CTRL at T50, but it took longer time for those exposed to QN. Even at T80, a good proportion of pearl oysters still were less active than CTRL and some other drug trials. However, it would be inexact to consider these end values as probable standard rate for the species because particular studies should be previously applied. This information is primary when the physiologic response of an organism to environmental changes is measured or interpreted through some index of stress over time. In marine bivalves, stress has been explained by functions such as clearance and filtration rate, heart beat, components of excretion, oxygen consumption, and so forth. (Bayne et al. 1976a, Bayne et al. 1976b). This last function is implicit in the recovery index used

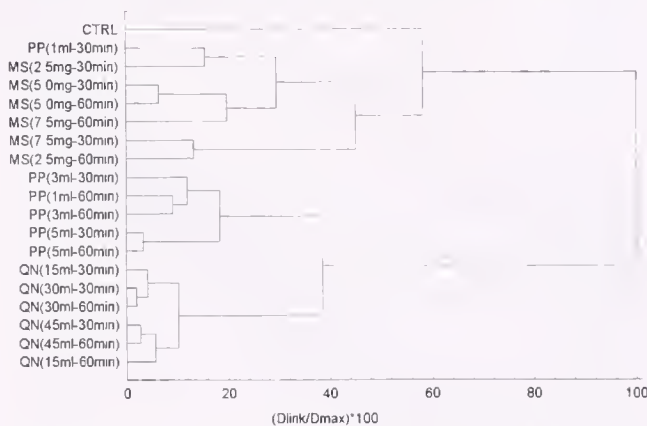


Figure 2. Hierarchical tree with multiple covariance analyses of experimental datasets and CTRL. Clusters are defined by Euclidian distances and complete linkage.

in this work. Bayne et al. (1976a) stated that *Mytilus edulis* under starvation rapidly arrives to standard respiration rate when gametogenesis is completed and energy reserves of the body are at the minimum. In this context, we assumed that all individuals entered the current experiment at a similar level of activity. Nevertheless, the comparative information of oxygen consumption in bivalves is controversial due to the variety of measurement methods, units used, and experimental designs (Shumway 1982). Concerning pearl oysters, Yukihiro et al. (1998) reports R_r (R in the cited work) of 0.857 and 1.039 mL O_2 oyster $^{-1}$ h $^{-1}$ in *P. maxima* and *P. margaritifera*, respectively, as intercept values from a significantly inclined and positive regression of R_r , or R , over dry weight. The authors considered these species as exceptional in having the highest indexes of energy flux recorded for bivalves. Saucedo et al. (2003) defined respiration rate of 0.4 to 0.8 $\mu\text{mol g}^{-1}$ h $^{-1}$ in fed and acclimatized *P. mazatlanica* between 18°C and 33°C. With these considerations, presurgery treatments in *P. mazatlanica* are difficult to explain in terms of physiologic response, and this lack of information calls for such studies. Aside from the intrinsic effect of chemicals even into complex body functions (Wagner et al. 2000; Eversen et al. 2003), the induction surgery would initiate hardly known immunologic processes in pearl oysters (i.e., Ortuño et al. 2002 in *Sparus aurata*). Norton et al. (2000) associated the use of PP with total failures in round pearl induction on *P. margaritifera* (mortality + nucleus rejection).

CONCLUSIONS

The best therapies in *P. mazatlanica* should sustain a nearly horizontal slope and low R_r -intercept during recovery; homogeneity of individual response is also important. These observations may comply with other species of pearl oysters. In real practice, the use of QN at doses of 45 mL eased the surgery for round pearl due to adequate and longer relaxation of specimens, but protection equipment is recommended. Treatments with 5 mL of PP were also acceptable, which suggest that higher dose and/or exposure time under this drug would provoke a "QN"-like response profile in this species, probably with shorter recovery time. Doses of MS-222 between 5 and 7.5 mL at 60 min of exposure could be used in *P. mazatlanica* for Mabé implants only. All specimens treated with this therapy had reasonably relaxed their abductor muscle, and mantle retraction was none or very slow; however, we noted about 70% of them arriving with contracted gonadal tissue and ligaments and/or foot muscle, which introduced a problematical approach to the insertion site. Based on our results, there is no evidence that longer exposure and/or higher dose of MS-222 might lead to low-activity profiles. Aside from the need for studies regarding the physiologic response profiles and relationship with functions of body parts, tolerance tests to these three drugs are important. In this experiment, mortality could not be attributed to any drug into the effective ranges of therapies. Incidence of mortality in field culture suggest handling effects.

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EFFECTS OF SALINITY AND TURBIDITY ON THE FREE AMINO ACID COMPOSITION IN GILL TISSUE OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*

NAM-HYUN LEE,¹ KYUNG-NAM HAN¹ AND KWANG-SIK CHOI^{2,*}

¹Department of Oceanography, College of Natural Science, In-Ha University, 253 Yonghyun-Dong, Nam-Gu, Incheon 402-751, KOREA; ²School of Applied Marine Science, College of Ocean Science, Cheju National University 1 Ara I-Dong, Jeju, 690-756 Korea

ABSTRACT Effects of salinity and turbidity stresses on free amino acid (FAA) composition in *Crassostrea gigas* was investigated using high performance liquid chromatography. Oysters were exposed for 2 to 5 days to low salinity (LS, 7‰), normal (control, 30‰), high salinity (HS, 39‰), and high turbidity in normal salinity (HT, 30‰, 0.326 g kaolin clay/l). Levels of aspartic acid, glutamic acid, serine, glycine, β -alanine, taurine, and L-alanine in the gill tissues were then monitored using a HPLC. Taurine and glycine were the two major contributors to the total FAA in gill tissues of oysters collected from fields, ranging from 200.7–324.7 and 42.9–69.0 μ mol/mg dry tissue. On day 2, total FAA in HS and HT was significantly higher than the levels in the control and LS; taurine and glycine levels in HS and HT were significantly higher than the levels of the controls and LS ($P < 0.05$). In contrast, most FAA levels in LS had dropped except for glutamic acid, glycine, and β -alanine; β -alanine level of oysters in LS on day 2 was about 10-fold higher than the control. Aspartic acid and Glutamic acid levels in HT on day 2 were significantly higher than the level observed in HS, LS and control ($P < 0.05$). From day 2 to 5, total FAA in the control increased 11.4% whereas total FAA in HS, LS, and HT decreased 17.3%, 21.6%, and 19.3% respectively. Aspartic acid and glutamic acid levels in the control also increased 29.1% and 50.6% between day 2 and 5 whereas the levels in HS, LS, and HT dropped dramatically, from 47.1 (HT) to 72.0% (LS) in case of aspartic acid and 53.5 (HS) to 68.5% (LS) in glutamic acid. Response of FAA composition in the gill tissues to salinity and turbidity stress was unique in this study, suggesting that FAA analysis is a useful tool to diagnose environmental stress to *C. gigas*, as was observed in *C. virginica*.

KEY WORDS: free amino acids, *Crassostrea gigas*, salinity, high turbidity, stress, HPLC, oyster

INTRODUCTION

Marine invertebrates contain very high levels of free amino acids (FAA) in their tissues and fluids, being 100 to 1000 times higher than those found in the extra-cellular fluid (Gilles 1972; 1979; Bayne et al. 1979). The FAAs are involved in a wide variety of biochemical and physiologic processes, and they are often monitored as a stress indicator in marine invertebrates under natural or man-made disturbances (Lynch & Wood 1966; Briggs 1979; Powell et al. 1984; Burton 1992). Epidermal tissues of marine bivalves in particular contain high level of FAAs, and the level of FAAs in the tissues often indicates their physiologic and nutritional status of an animal (Feng et al. 1970; Biondi & Novello 1978; Sakaguchi & Murata 1989; Paynter et al. 1995). It is also well known that intracellular FAA in bivalve tissues are used in cellular volume regulation. An increase or decrease in salinity often results in an increase or decrease of FAA level in the tissues (Lynch & Wood 1966; Baginski & Pierce 1977; Shumway & Gabbott, 1977; Powell et al. 1982; Ellis et al. 1985).

C. gigas is an estuarine bivalve inhabiting intertidal to shallow subtidal areas in Korea, Japan, and China where they are commercially raised. The oysters are particularly abundant in coastal estuarine areas where fluctuations of temperature and salinity, as well as man-made pollution, often exert significant physiologic impacts (Feng et al. 1970; Livingston 1985; Tirard et al. 1997). *C. gigas* tissues are enriched with amino acids, and the levels of free amino acids in the tissues often reflect environmental changes as well as seasonal variations (Sakaguchi & Murata 1989). Although there are many reports on the effects of environmental changes on *C. gigas* physiology as measured with various biochemical assays, studies on the effects of environmental changes on the FAA pool are rare despite the commercial importance of this species. This

study attempts to evaluate the effects of salinity and turbidity on the FAA pool of Pacific oysters.

MATERIALS AND METHODS

Oysters used in the experiment were collected from Kamakman Bay on the south coast of Korea, where the oysters are commercially cultured using the hanging culture system. Salinity in this bay fluctuates between 28‰ and 33‰, and the visibility of the water varies from 1.5–3 m annually (Hyun et al. 2001). Oysters of 8–12 cm long and 10–15 g wet tissue weight were collected from oyster grow-out hanging lines placed at a depth between 1 and 4 m below the surface. The gill tissues of 13 oysters were excised *in situ* and frozen immediately using dry ice and subjected to FAA analysis. The amino acid composition of the control oysters collected from Kamakman Bay were compared with that of other oysters collected from an intertidal area at Daechon on the west coast of Korea where salinity varies 25‰ to 35‰ annually.

For the experiment, oysters were placed in static 50-L glass tanks filled with normal salinity seawater (30‰). After a 2-day acclimation period, the following experimental conditions were applied: (1) control at 30‰; (2) high salinity stress at 39‰ (HS); (3) low salinity stress at 7‰ (LS); and (4) high turbidity (HT) stress created with kaolin-clay with a constant concentration of 0.323 gm/l in 30‰ seawater according to Powell et al. (1982). A set of 20 oysters was placed in each 50-L glass tank, and the water was continuously stirred and aerated during the experimental period. Seawater in the tanks underwent a 100% water change twice daily. To maintain constant high turbidity in the tank, the kaolin concentration was checked four times a day and a proportional amount of clay was added if necessary.

On days 2 and 5, seven oysters were collected from each experimental tank to measure FAA in their gill tissues. Oysters were opened and gill tissues removed, weighed, and stored at –70 °C until analyzed. Dry weight of the gill tissue was estimated from the

*Corresponding author. E-mail: skchoi@cheju.ac.kr

empirical relation; dry weight = wet weight of gill tissue \times 0.196 (Choi et al. 1993). The oyster gill tissue was homogenized in phosphate buffer (pH 7.3) using a mortar and ultrasonifier at 4 \square . The homogenate was centrifuged and the supernatant collected for analysis. The supernatant was then treated with trichloric acid (TCA), from 10%–50%, to precipitate proteins and nucleic acids (Powell et al. 1982). TCA treated samples were centrifuged to remove protein and the supernatant containing FAA was harvested. TCA in the supernatant was then removed using six applications of ether. Types of FAA in the samples were determined with HPLC using methanol-acetate as a solvent. Orthophthaldialdehyde (OPA) was used as the detecting compound for identification and quantification of FAA. Aspartic acid, glutamic acid, serine, β -alanine, L-alanine, glycine, and taurine were analyzed from the FAA pool in the oyster gill tissues and finally expressed as μ mol FAA/g dry tissue weight.

RESULTS

FAA Composition of Oysters Collected *In Situ*

Amino acid composition in gill tissues of control oysters is summarized in Table 1. Among the seven amino acids analyzed, taurine and glycine were the most abundant amino acids in the gill tissues sampled from the Daechon and Kamakman Bay. These two amino acids accounted for more than 70%–80% of the free amino acids measured in the oysters.

Changes in FAA Composition in Oysters in Control Tank

Table 2 summarizes changes in the FAA composition of oysters held in the control tank (30‰) for 2 and 5 days. As observed in wild oysters, taurine and glycine were the most abundant compounds that accounted for more than 82% of total FAAs in oysters in the control tank for 2 days. In addition to taurine and glycine, L-alanine, aspartic acid, and glutamic acid were present in the oyster tissues at levels of 20.14, 16.72, and 14.23 μ mol/g dry tissue weight respectively. In contrast, serine and β -alanine levels in the control oysters were <10 μ mol/g dry tissue. After 5 days in the control tank, glutamic acid levels increased by 50.6% relative to the level at day 2. In contrast, β -alanine and L-alanine levels at day 5 dropped by 31.1% and 32.5%.

FAA Changes in HS Tank

All FAA levels in oysters in the increased salinity at day 5 were lower than the FAA levels in oysters at day 2, except serine.

TABLE 1.

FAA composition of oyster gill tissues collected from Kamakman Bay and Daechon (N = 26). Numbers in parenthesis represent standard deviation. Unit = μ mol/mg dry tissue weight.

	Daechon	Kamakman Bay
Aspartic acid	16.5 (2.5)	18.5 (2.1)
Glutamic acid	28.3 (7.7)	34.2 (6.8)
Serine	13.2 (1.5)	10.1 (1.7)
Glycine	69.0 (20.6)	42.8 (8.7)
β -Alanine	15.9 (3.3)	5.2 (0.1)
Taurine	200.7 (48.3)	324.4 (45.1)
L-Alanine	37.3 (11.0)	29.7 (7.2)
T/G ratio	2.9	7.6
TOTAL	380.8 (91.9)	464.8 (60.6)

Aspartic acid, glutamic acid, glycine, and β -alanine levels dropped by –54.1%, –53.5%, –55.8% and –36.1% respectively. Contrary to other FAAs, taurine levels between day 2 and day 5 remained largely unchanged (see Table 2). Due to the taurine, total FAAs had only dropped –17.3% between day 2 and day 5 in the high salinity tank.

Compared with the control oysters, total FAA levels in oysters held in HS tank for two days were found to be 1.61 fold elevated (Table 3). Particularly, the glycine level in the oysters at Day 2 was much higher, by a factor of 2.90, than the level in oysters held in the control tank (30‰) at day 2. It was also noticed that the β -alanine level in HS at day 2 was 3.05-fold higher than the level in oysters held in the control. The taurine level of oysters in the tank was also 1.47-fold higher than the level of control oysters. In contrast, the serine level remained largely unchanged at day 2. By day 5, aspartic acid and glutamic acid levels had dropped by a factor of 0.60 relative to the control oysters. However, the β -alanine level of the oysters in the LS tank was much higher, a factor of 2.83, than the level in the control salinity tank. Serine, glycine, and L-alanine levels were also higher than the control oysters at day 5, by a factor of 1.70, 1.53, and 1.63 (see Table 3).

FAA Changes in LS Tank

Between day 2 and day 5 in the LS tank, all FAA levels in the oysters had dropped. Aspartic acid, glutamic acid, glycine, and β -alanine levels fell by 72.0%, 68.6%, 34.6%, and 31.9%. In contrast, taurine, that accounts for more than 70% of the total FAA in the oysters, decreased by only 8.8% (see Table 2).

After 2 days in LS (7‰), the taurine level dropped by a factor of 0.68 compared with the level of the control oysters (see Table 3). Aspartic acid and L-alanine levels in the oysters also decreased by factors of 0.59 and 0.46 relative to the control oysters at day 2. However, the glutamic acid and β -alanine levels were elevated by a factor of 1.85 and 10.36 respectively. At day 5, the total FAA level dropped by a factor of 43% compared with the control oysters at the same time period. Aspartic acid and glutamic acid levels dropped by a factor of 0.13 and 0.39 relative to the control oysters. In contrast, the β -alanine level in oysters at day 5 remained elevated as observed at day 2, 10.23-fold higher than the β -alanine level in the control oysters (see Table 3).

FAA Changes in HT Tank

Between day 2 and day 5, most FAA levels in the oysters dropped more than 30%. Aspartic acid, glutamic acid, glycine, and β -alanine levels dropped by 47.1%, 64.1%, 36.6%, and 30.1% respectively. However, the serine level had risen by 55.9% after 3 days in HT, between days 2 and 5 (see Table 2).

Oysters incubated in the HT tank for 2 days showed a very high level of taurine compared with the control oysters. Taurine level of the oysters was 1.85-fold higher than the level of the control oysters. Other FAA levels were also elevated by more than 30% for 2 days (see Table 3). Aspartic acid and glutamic acid levels also increased by a factor of 2.40 and 2.78 relative to the control oysters. Total FAAs of the oysters were also 1.81-fold higher than the total FAAs of the control oysters at day 2. At day 5, total FAA level in the high turbidity tank remained elevated by a factor of 1.31. The taurine level of the oysters at day 5 was 1.38-fold higher than the taurine level of the control oysters. In contrast, glutamic acid levels in the oysters at day 5 were lower than those of the control oysters by a factor of 0.66 for the same length of time.

TABLE 2.

Concentration of amino acids and their percent changes in the FAA pool in controlled and treated oysters at two and five days. Each concentration value represents the mean of seven samples (unit = μmol FAA/g dry tissue). % change = (Day 5/Day 2) - 1.

Amino Acid	Normal (Control, 30‰)			High Salinity (HS, 39‰)			Low Salinity (LS, 7‰)			High Turbidity (HT, 0.326g kaolin clay/l)		
	Day 2 (N = 7)	Day 5 (N = 7)	% Change	Day 2 (N = 7)	Day 5 (N = 7)	% Change	Day 2 (N = 7)	Day 5 (N = 7)	% Change	Day 2 (N = 7)	Day 5 (N = 7)	% Change
Aspartic Acid	16.7	21.6	29.1	28.1	12.9	-54.1	9.8	2.8	-72.0	40.1	21.2	-47.1
Glutamic Acid	14.2	21.43	50.6	27.5	12.8	-53.5	26.3	8.3	-68.5	39.6	14.2	-64.1
Serine	8.2	6.19	-24.0	9.2	10.5	14.9	6.6	4.9	-25.3	8.9	13.9	55.9
Glycine	26.9	22.60	-15.9	78.0	34.5	-55.8	31.7	20.7	-34.6	34.9	22.1	-36.6
Taurine	260.5	301.57	15.8	382.1	367.4	-3.8	177.8	162.2	-8.8	483.0	417.5	-13.6
β -Alanine	2.2	1.51	-31.1	6.7	4.3	-36.1	22.7	15.5	-31.9	3.5	2.5	-30.1
L-Alanine	20.1	13.60	-32.5	29.91	22.1	-26.0	9.3	8.3	-10.7	20.6	17.7	-14.0
Total Less Taurine	88.3	86.9	-1.6	179.3	97.1	-45.9	106.4	60.5	-43.2	147.6	91.6	-37.9
Total	348.8	388.5	11.4	561.4	464.4	-17.3	284.2	222.7	-21.6	630.5	509.0	-19.3

DISCUSSION

FAA Composition in Field Oysters

Table 1 indicates that taurine is the most abundant FAA in the control oysters collected from Kamakman Bay, followed by glycine. Taurine and glycine account for 52% and 18% of total FAAs respectively in oysters collected from Daechon. Sakaguchi and Murata (1989) also reported that taurine and glycine are the most abundant FAAs in cultured *C. gigas*. High levels of taurine in the FAA pool also have been reported from various marine bivalves; it is believed that most marine bivalves use taurine in osmotic volume regulation (Sansone et al. 1978; Powell et al. 1982; Lynch & Wood 1966; Feng et al. 1970). Taurine levels in oysters col-

lected from Kamakman Bay were much higher than the levels observed in Daechon oysters, whereas the glycine level of Kamakman Bay oysters was lower than those from Daechon. Taurine comprises 70% of the total FAA, whereas glycine accounts for only 9% in Kamakman oysters.

Different FAA levels observed between Kamakman Bay and Daechon oysters could be associated with different levels of tolerance to stress and anoxia during transportation to the laboratory. Daechon oysters were collected from the intertidal zone where the oysters are exposed to the atmosphere twice a day during low tide. In contrast, Kamakman Bay oysters were collected from an oyster farm where oysters were hanged on suspended rope and submerged from 2-5 m below the surface. It is likely that the intertidal oysters from Daechon are better adapted to anoxic condition formed during transportation by closing their valves. Several studies have reported that marine bivalves often exhibit elevated levels of taurine due to an intracellular response to change in extracellular fluid osmolarity (Pierce 1971; Powell et al. 1982; Paynter et al. 1995).

TABLE 3.

Pair-wise comparisons between all treatments with amino acids at Day 2 or 5 listed below each pair. Each number represents a ratio between mean of one treatment to the other

	Oysters Day 2		
	High Salinity vs. Normal	Low Salinity vs. Normal	High Turbidity vs. Normal
Aspartic Acid	1.7	0.6	2.4
Glutamic Acid	1.9	1.9	2.8
Serine	1.1	0.8	1.1
Glycine	2.9	1.2	1.3
Taurine	1.5	0.7	1.9
β -Alanine	3.1	10.4	1.6
L-Alanine	1.5	0.5	1.0
Total Less Taurine	2.0	1.2	1.7
Total FAAs	1.6	0.8	1.8
	Oysters Day 5		
	High Salinity vs. Normal	Low Salinity vs. Normal	High Turbidity vs. Normal
Aspartic Acid	0.6	0.1	1.0
Glutamic Acid	0.6	0.4	0.7
Serine	1.7	0.8	2.3
Glycine	1.5	0.9	1.0
Taurine	1.2	0.5	1.4
β -Alanine	2.8	10.2	1.6
L-Alanine	1.6	0.6	1.3
Total Less Taurine	1.1	0.7	1.1
Total FAA	1.2	0.6	1.3

Effects of Changes in Environmental Factors on FAA Composition of Oysters

Man-made disturbances such as high concentrations of heavy metals, hydrocarbons, and drilling effluents, as well as natural stresses, such as starvation, decrease or increase in salinity, and parasitism, often alter the composition of FAAs in marine animals (Jeffries 1972; Pecon & Powell 1981; Roesijadi & Anderson 1979; Widows et al. 1982; Bayne et al. 1976; Kendall et al. 1985; Paynter et al. 1995) as well as the protein synthesis rate (Choi et al. 1994; Tirard et al. 1997). However, the response of the animals in terms of internal FAA change varies with different environmental or natural stresses. Hyper-osmotic, hypo-osmotic, and high turbidity stresses were applied to *C. gigas* in the present study. Oysters exposed to high salinity and high turbidity stress showed a remarkable increase in total FAA for the first 2 days (see Tables 2 and 3).

Other studies have also reported elevated FAA levels in marine bivalves exposed to hyper-osmotic conditions for a short period (Powell et al. 1982; Lynch & Wood 1966). *C. virginica* exposed to high salinity (38‰) for 2 days showed elevated total FAAs. Glycine, alanine, and β -alanine levels were elevated compared with the control oysters (Powell et al. 1982). *C. gigas* in this study also

showed a remarkable increase in glycine, L-alanine, and β -alanine levels when treated with high salinity stress. Baginski and Pierce (1977) also observed elevated alanine and glycine levels in *Modiolus demissus* (= *Geukensia demissa*) 1 to 3 days after holding the mussels in a hyper-osmotic environment. Accumulation of alanine and glycine during hyper-osmotic stress also has been reported for the mussels, *Mytilus edulis* and *Geukensia demissa* (Deaton et al. 1985; Deaton 2001). The increased FAA levels observed in the oysters held in the high salinity tank for the first 2 days are due to volume regulation by the cells of the oysters as reported in other marine bivalves (Deaton et al. 1985).

Oysters exposed to high turbidity water for 2 days also showed a remarkable increase in the FAA levels. Compared with the control oysters, all levels of FAAs in the gill tissue of those oysters were elevated for the 2 days of immersion in highly turbid water. However, the pattern of elevation in FAA is somewhat different between oysters treated with high salinity and high turbidity stress. Taurine, aspartic acid, and glutamic acid levels in the oysters in the high turbidity tank for 2 days were remarkably elevated by a factor of 1.85–2.78 relative to the FAA level in control oysters for the same length of time (see Table 3). In contrast, glycine and β -alanine levels in oysters held in the high turbidity tank for 2 days increased by only a factor of 1.30 and 1.61 respectively. As shown in Tables 2 and 3, the taurine level in oysters held in the high salinity tank for 2 days increased only a factor of 1.47, whereas glycine and β -alanine levels in oysters held in this tank for the same length of time were 2.90- and 3.05-fold higher than the levels in control oysters.

Powell et al. (1982) also observed elevated FAA levels in *C. virginica* held for 2 days in a high turbidity tank containing kaolin clay as turbid particles. Particularly, the oysters held in the kaolin-clay tank showed a remarkable increase in taurine and cystic acid levels. One possible explanation for the observed increase in taurine level in the oysters in this study would be the elevated mucus secretion activities. The oysters in the high turbidity tank would secrete more mucus to remove the kaolin clay particles captured in the gills. According to Powell et al. (1982), mucus of marine invertebrates, including oysters, contains high level of sulfate anion. An increase in mucus secretion in oysters may result in cystic acid levels in the FAA pool because an increase in sulfate anion level in the mucus also elevates a precursor compound for cystic acid. Powell et al. (1982) also observed that an increase in cystic acid levels accompanies an increase of taurine levels in their study. Thus, we postulate that, in this study, the observed increased taurine levels in the oysters in the high turbidity tank on day 2 was influenced by elevated cystic acid levels in the mucus, although cystic acid was not analyzed in this study.

Oysters in hypo-osmotic conditions for 2 days exhibited slightly decreased total FAA levels in the gill tissues. As shown in Table 2, aspartic acid, taurine, and L-alanine levels in the oyster gill tissues dropped more than 30% compared with the level of control oysters. In contrast, glutamic acid and β -alanine levels in oysters in low salinity conditions were much higher than the levels observed among control oysters. Especially, the β -alanine level in the oysters was 10 times higher than the level in the control oysters, and 3–5 times higher than the level in oysters held in the high

salinity or high turbidity tanks. Although the β -alanine levels showed dramatic changes after 2 days in the tank, the role of β -alanine in the FAA pool's response to osmotic stress seems to be minor; β -alanine accounts only for 8% of the total FAA. Elevation of β -alanine levels was also observed in *C. virginica* held in increased salinity, anoxia, drilling effluent from oil drilling operations, and high turbidity treatments for 5 days (Powell et al. 1982). An increase in β -alanine levels was also reported from sea anemones raised in a laboratory and in the field (Low et al. 1980). It is likely that the elevation of β -alanine observed in *C. gigas* is a unique physiologic response to stress, although the mechanism of β -alanine elevation in the FAA pool of either *C. virginica* or *C. gigas* is not fully understood.

FAA levels in oysters in the experimental tanks at day 5 were considerably decreased compared with either the day 5 controls or the day 2 treatment groups (see Table 2). Decreases in FAA between days 2 and 5 were very conspicuous in the oysters held in the low salinity tank. All amino acids analyzed in the oysters exhibited an 8.8%–72% decrease in FAA between days 2 and 5. Specifically the levels of glutamic acid declined dramatically between those two days. Those levels dropped 53.5%, 68.5%, and 64.1% respectively in the high salinity, low salinity, and high turbidity tanks. However, it is unlikely that the dramatic changes in glutamic acid observed in this study exert a great influence on osmotic balance of the oysters since glutamic acid is a minor constituent of the FAA pool. Glutamic acid accounts for only 2.8%–5.5% of the total FAA at day 5. In contrast, taurine, which comprises from 62.6%–82.0% of the total FAA pool in the oysters at day 5, remained less changed compared with other amino acids.

A considerable decrease in FAA was also observed in *C. virginica* held in drilling effluent, anoxia, and high turbidity tanks between days 2 and 5; the oysters exhibited a remarkable decrease in FAA, including taurine, glycine, and alanine (Powell et al. 1982). Particularly, the taurine level in the oysters dropped as much as 58% in the anoxia tank between days 2 and 5. Powell et al. (1982) postulated that such a considerable drop in amino acid levels in *C. virginica* could be attributed to a general loss of FAA from the gills. This was because of some changes in the tissue's ability to prevent FAA loss across the outer membrane of the cells in the gill tissues, as indicated by another study (Bishop et al. 1994). Although the extent of decrease in the taurine level in *C. gigas* was not as great in as *C. virginica*, the oysters held in the kaolin turbidity tank, in this study, also showed a remarkable decrease in the taurine level between days 2 and 5. It is believed that *C. gigas*, in the kaolin tank, also loses a large amount of taurine through the gills, as observed in *C. virginica*.

ACKNOWLEDGMENTS

We are grateful to E. N. Powell of Haskin Shellfish Laboratory of Rutgers University and Charles Bai of Pukyong National University for reviewing and commenting on the manuscript. This work was supported at the regional research center for coastal environmental of Yellow Sea at Inha University designated by Ministry of Science and Technology (MOST) of Korea and Korea Science and Engineering Foundation (KOSEF).

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THERMOTOLERANCE AND HSP70 PROFILES IN ADULT AND EMBRYONIC CALIFORNIA NATIVE OYSTERS, *OSTREOLA CONCHAPHILA* (CARPENTER, 1857)

HEATHER M. BROWN,^{1,2} ALISON BRIDEN,¹ TARYN STOKELL,¹ FRED J. GRIFFIN^{1*} AND GARY N. CHERR^{1,3}

¹Bodega Marine Laboratory, PO Box 247, Bodega Bay CA, 94923 ²Current address: Department of Molecular, Cellular and Developmental Biology, University of California, Santa Cruz, CA, 95064

³Departments of Environmental Toxicology and Nutrition, University of California Davis, Davis CA, 95616

ABSTRACT The oyster *Ostreola conchaphila* is endemic to shallow subtidal and low intertidal marine waters of the West coast of North America. The heat shock response of the species was characterized for both adult and early life stages. *O. conchaphila* expressed proteins that were the same molecular weights as and were immunologically related to the heat shock protein (Hsp)70 family of proteins that have been described for the oyster, *Crassostrea gigas*. Hsp77 and Hsp72 were present in adult and larval tissue. Expression of Hsp69 occurred in adults that were heat shocked at 33–38°C, regardless of season or previous environmental temperature regimen. A heat shock at 34°C induced thermotolerance to otherwise lethal temperatures of 38.5° or 39°C. Early life stage embryos (early cleavage, late cleavage, and ciliated blastula stages) were unable to express Hsp69 or mount a heat shock response. Veliger larvae expressed Hsp69 after a 34°C heat shock. The increased Hsp69 persisted for at least 12 h after 1 h of heat shock as opposed to the acquisition of thermotolerance, which had declined by 12 h after heat shock.

KEY WORDS: Hsp70, thermotolerance, larvae, Olympia oyster, Native oyster, *Ostrea conchaphila*

INTRODUCTION

An important component of tolerance to stress at the cellular level involves the “heat shock response.” This response involves changes in the levels of several families of heat shock proteins (Hsps) or molecular chaperones that stabilize protein conformation and function in the face of denaturing stress, including thermal stress, thus providing protection (Lindquist 1986, Gething & Sambrook 1992, Morimoto et al. 1994). Typically part of the response involves selective expression of certain inducible Hsps, which in turn can provide an enhanced tolerance beyond lethal conditions (Parsell & Lindquist 1994, Mailhos et al. 1993, 1994). Three Hsp isoforms exist; two are constitutive, Hsp77 and Hsp72, and are routinely present in cells, whereas the expression of a third, Hsp69, is induced after thermal shock (Clegg et al. 1998, Feder & Hoffman 1999). The Hsp70 family is directly implicated in thermal tolerance of several marine invertebrates, however, the temperature at which the heat shock response is activated not only differs between species, but can also vary within species as a result of environmental or physiologic history. For example, the temperature for induction (set point) of the heat shock response in the intertidal mollusks within the genera *Tegula*, *Mytilus*, and *Crassostrea* is a function of acclimatization to past thermal conditions (Tomanek & Somero, 1999, Buckley et al. 2001, Hamdoun et al. 2003). And, in at least one genus, *Crassostrea*, there is a cost to this acclimation; this increase of the Hsp induction set point is not balanced by an equivalent increase in the thermal tolerance limit (Hamdoun et al. 2003). Thus, thermally acclimatized *Crassostrea* may be less fit to tolerate stress.

The North American Olympia oyster, *Ostreola conchaphila*, has been characterized as intolerant to both tidal exposure and temperature stress. A native to the coast of western North America from Sitka, Alaska to Panama (Harbo 1997), this comparatively small (<60 mm) and slow growing species is found attached to rock or other hardened substrates in bays and estuaries extending

from low intertidal to shallow subtidal depths (Baker 1995). Like other oysters of the genus *Ostrea* and *Ostreola*, fertilization and embryonic development in *O. conchaphila* take place in the mantle cavity of females, thus early life stages are captive to the same environmental conditions as brooding adults. Hopkins (1937) reported that *Ostrea lurida* (*O. conchaphila*) could not withstand temperatures outside the range of 0–30°C and this limited thermal tolerance range was described as the primary constraint to geographical distribution of the species. This upper thermal limit is considerably lower than that reported for its European congener, *Ostrea edulis* (40°C) or the Japanese oyster, *Crassostrea gigas* (43–44°C), both of which inhabit intertidal to shallow subtidal environments, but do so over a broader geographical range (Clegg et al. 1998, Piano et al. 2002).

In the present report, we characterize the heat shock response for *O. conchaphila* adults and veliger larvae from Tomales Bay, California. We present evidence for the induced expression of Hsp 69 after thermal stress, demonstrate that the response is short-lived, but does convey thermal tolerance, and discuss the ecological significance of this response. We present evidence that the response is not present in pre-gastrula embryos and appears to be more truncated in larvae than in adults. Lastly, we address the question: Is the Hsp70 family expression pattern in *O. conchaphila* genetically fixed and how does this response compare with the phenotypic plasticity observed in *C. gigas* and other marine invertebrates?

MATERIALS AND METHODS

Animal Collection and Maintenance

Adult oysters were collected from Tomales Bay, California, at a tidal height of 0–1.0 ft and transported to the Bodega Marine Laboratory within 1–2 h of collection. Animals were maintained in flow-through seawater tanks with aeration at 12–15°C and were fed an algal paste daily consisting of *Chaetoceros*, *Tetraselmis*, and *Isochrysis* (Reed Mariculture, Inc). Collections were conducted during June through July over successive years (2000 and

*Correspondence: tjgriffin@ucdavis.edu

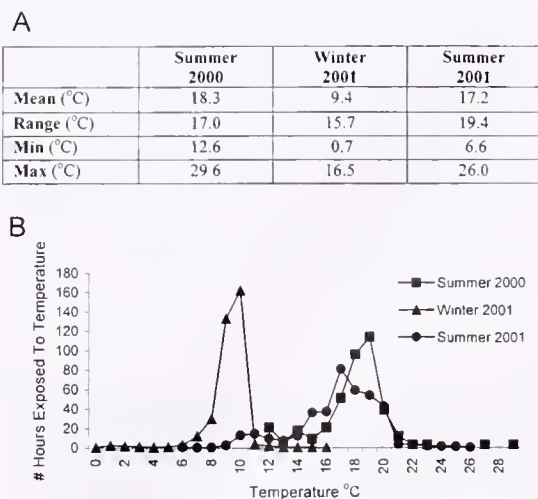


Figure 1. Temperature profile for Tomales Bay. Hobo samplers were used to collect temperatures at the tidal height and location of *O. conchaphila* for a 2-week period prior to each collection. A, Temperature range (range), mean, minimum (min), and maximum (max) temperatures were calculated for each site. B, In addition, the total number of hours at each temperature (rounded to the nearest °C) over the 2-week period was calculated.

2001), with one collection during December 2000 for seasonal comparison. Adults were used in heat shock experiments within 3–5 days of collection. During recovery periods after heat shock, adults were maintained in the flow-through seawater tanks and fed as described above.

Embryos and larvae were obtained from brooding females collected during the summer; the reproductive season in northern California extends from May to September, during which time females brood embryos for the first 10–12 days of embryonic development (Bonnot 1935, Baker 1995). To remove the larvae from females, the right valve of the shell was removed and the mantle cavity was rinsed with 0.45- μ m filtered seawater (FSW) over an 85- μ m mesh screen to filter out large debris. The filtrate, containing embryos or larvae was then passed through a 20- μ m filter to collect and concentrate the larvae. Collected embryos or larvae were cultured in aerated FSW, 15°C, in 20 \times 11 \times 6-cm glass dishes at a concentration of approximately 200 embryos per

milliliter. All experiments with embryos and larvae were conducted within 1 day of collection during which time the larvae were cocultured with *Isochrysis* as a larval food source.

Temperature Data Logging

Tomales Bay temperature data from the oyster collection site was obtained using a Hobo® Tid-Bit temperature sampler that was placed at a tidal height of 0 ft. Temperature readings were recorded at 2-min intervals between May and September 2001.

Heat Shock and Lethal Temperature Determination

For all adult heat shock experiments, animals ($n = 10$) were removed from ambient seawater and placed for one hour into pre-heated seawater in a 2L beaker that was, in turn, placed in a Neslab RTE211 waterbath that set to within 0.1°C of the experimental temperature. Adult oysters were heat shocked at temperatures ranging in one-degree increments from 32 to 39°C. After heat shock, adults were transferred back to ambient seawater, with daily feeding, to recover. Mortality was monitored daily for two weeks.

Embryo and larval heat shock experiments were conducted at a concentration of 200 embryos/ml in 20-mL scintillation vials by suspending the vials in the Neslab RTE211 water bath for one hour at the appropriate temperature. Four stages of development, early cleavage, late cleavage, ciliated blastula, and veliger larvae were used in the embryo/larval heat shock experiments. Embryos and larvae were provided a 24 h post-heat shock recovery period (monitored at 4 h, 12 h, and 24 h) at 15°C, then percent mortality was scored using a dissecting microscope. The criteria for viability vs mortality was stage dependent; early cleavage embryos were considered alive if cleavage had continued post-heat shock, while motility was used as the criteria for viability in embryos/larvae in the other three stages.

Hsp70 Determinations

The presence and quantities of Hsp70 proteins were determined by using oyster proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an Hsp70 monoclonal antibody. All procedures for sampling tissue and preparation for Hsp analyses were conducted according to Clegg et al. (1998) with modifications after Hamdoun et al. (2003). Briefly, gill samples were excised 48 h postheat shock, weighed, frozen in liquid nitrogen, and stored at -80°C. For electrophoresis, the pre-weighed gill tissue was placed in potassium gluconate buffer (KGB: 5 mM MgSO₄, 5 mM NaH₂PO₄, 40 mM Hepes, 70 mM gluconic acid, 150 mM sorbitol, pH 7.5) at a concentration of 100 mg wet tissue per 1 ml of KGB. Samples were then homogenized and diluted 1:1 with 2X SDS-PAGE sample buffer and heated to 100°C for 5 min. Volumes of solubilized gill were adjusted so that equivalent gill weights for each sample were loaded onto a 10% polyacrylamide gel, the proteins separated by SDS-PAGE, and protein bands subsequently transferred to nitrocellulose. Note: gels were electrophoresed for 30 min after the tracking dye had mi-

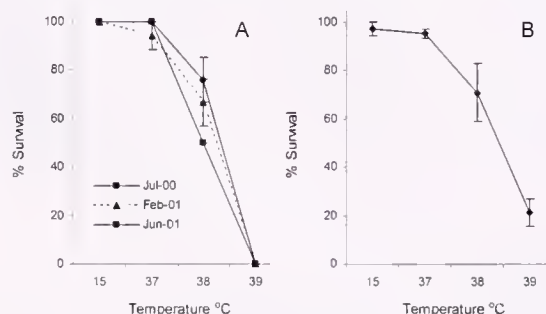


Figure 2. Lethal temperature profiles for adult and larval *O. conchaphila*. A, Survival of adults during summer 2000, winter 2001, and summer 2001 after a 1-h heat shock at 37, 38, or 39°C ($n = 18$ for each data point). There was no significant difference between seasonal survival at either 37 or 38°C ($P > 0.05$). B, Survival of veliger larvae, collected in July 2002, after a 1-h heat shock at 37, 38, or 39°C (200 larvae per ml; $n = 3$). Control adults and larvae were held at 15°C.

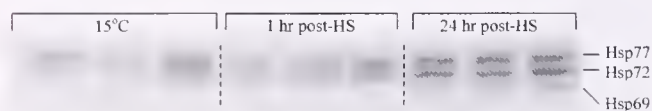


Figure 3. Immunoblots of Hsp70 protein profiles of control (15°C) and heat shocked *O. conchaphila* adults at 1 and 24 h post-heat shock (heat shock of 1 h at 34°C).

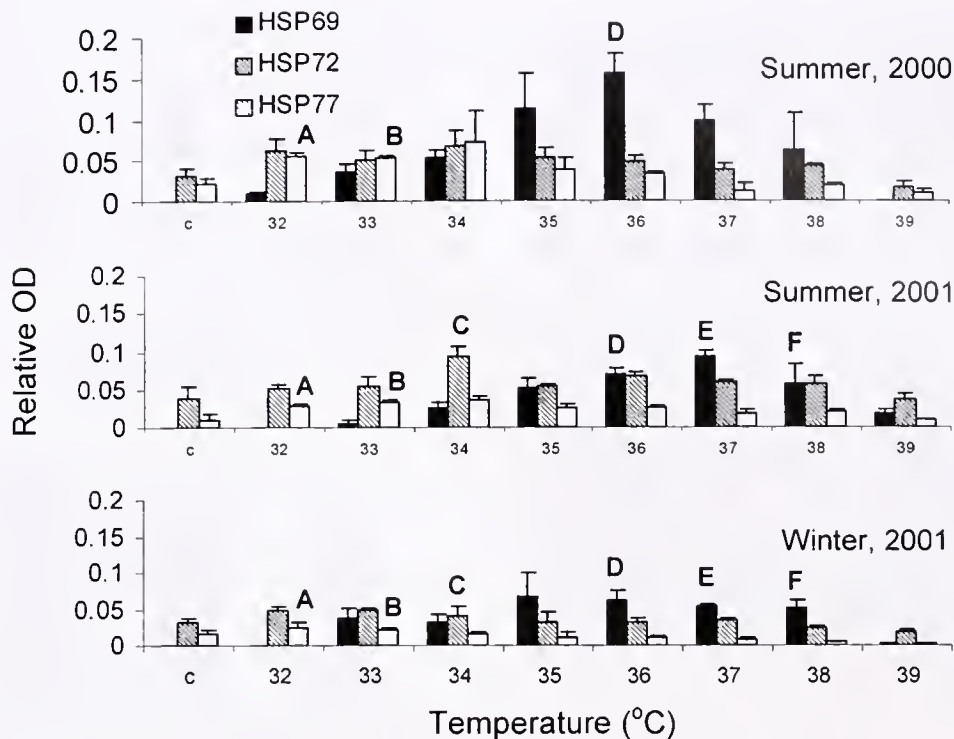


Figure 4. Hsp70 protein profiles of adult oysters after heat shock at 32–39°C. Relative levels of Hsp69 were assayed at 48 h post-heat shock in collected Summer 2000, Winter 2001 and Summer 2001. Seasonal profiles of Hsp69, 72, and 77 levels after heat shocks revealed no significant summer/winter differences (values are means \pm SEM ($n = 3$)). There were random significant differences (A-A, B-B, etc $P < 0.05$) that did not follow seasonal trends. Values were pooled and averaged \pm SEM ($n = 9$). Hsp69 levels after heat shock at temperatures ranging from 33–38°C (*) were significantly greater ($P < 0.05$) than 15°C controls or heat shock at either 32 or 39°C. Values in the seasonal data (inset) are means \pm SEM ($n = 3$ for each data set).

grated to the bottom of the gel; this provided separation of the Hsp70 isomers. Western blots were probed with a rat anti-HSP70 monoclonal antibody (MA3-001, Affinity Bioreagents) followed by a goat anti-rat horseradish peroxidase-conjugated secondary antibody (Sigma). Labeled proteins were detected using chemiluminescence (ECL, Pierce) in a UVP Epi Chemi II darkroom. Densitometry of the bands on western blots was conducted using Laboratory Works image acquisition and analysis software (UVP Bio-imaging Systems).

Larvae were sampled by removing 15–100 mg wet weight per time point from the culture vial and were concentrated on a 50- μ m nitex mesh screens. The larvae were washed with FSW, pelleted, frozen in liquid nitrogen, and stored at -80°C until all samples were collected. Prior to SDS-PAGE analysis, the larval pellets were resuspended in hypotonic lysis buffer (HLB: 10 mM KCl, 1.5 mM MgCl_2 , 10 mM Tris-HCl, pH 7.4) with protease inhibitors to 200 mg/mL, sonicated and then diluted 1:1 with 2X SDS-PAGE sample buffer and analyzed as stated above for the adults.

INDUCED THERMOTOLERANCE

Induced thermotolerance experiments followed procedures outlined in Clegg et al. (1998) with adjustments for species temperature tolerance differences. Adults received a sublethal heat shock (34°C , 1 h) and were allowed to recover at ambient for 24 h prior to a 1-h incubation at the preheat shock lethal temperature (38.5 or 39°C). Veliger larvae were subjected to a sublethal heat shock (34°C , 1 hr) and subsequently incubated at the pre-heat shock lethal temperature (39°C) at 6, 8, or 12 h postslethal heat shock. Con-

trols for both adults and larvae were exposed to 38.5 or 39°C without a prior sublethal heat shock treatment. Survival of adults was assessed over a 3-wk period and larvae survival was monitored over a 24 h period.

Data Analysis

Protein levels were analyzed for statistical significance using one-way analysis of variance (Sigmastat software v2.03).

RESULTS

Collection-Site Temperature Profiles

The temperature profiles at oyster collection sites in Tomales Bay for the 2 weeks leading up to each sampling are summarized in Figure 1. The profiles differed not only between winter and summer but also slightly between the two summer sampling periods. During the winter sampling, temperatures ranged from 0.7 to 16.5°C ; however, greater than 90% of the degree-hours (the number of hours at a particular temperature) for the 2-week period fell within a 3-degree range, 8 – 10°C (Fig. 1). During the summer there was more variation in water temperature; the ranges were 12.6 – 29.6°C and 6.6 – 26.0°C for 2000 and 2001, respectively. The temperature ranges that encompassed 90% of the degree hours for the 2-week summer periods were also broader, 16 – 20°C and 14 – 20°C , respectively.

Lethal Temperature Determination

The lethal temperature for *O. conchaphila* adult and larval life stages is between 38°C and 39°C . No adult oysters survived heat

shock for 1 h at 39°C, and the range of survival subsequent to a 1 h exposure to 38°C was 50–87%, with no statistically significant seasonal difference ($P > 0.1$; Figure 2A). Brooded veliger larvae (from summer collections) mimicked the adult pattern: $70.7 \pm 12.01\%$ SD (range of 59–83%) of larvae survived a heat shock of 38°C while only $21.3 \pm 5.7\%$ SD (range of 15–26%) survived 39°C (Fig. 2B).

Adult Hsp Protein Profiles

Ostrea conchaphila expresses the Hsp70 family of heat shock proteins; constitutive (Hsp77 and Hsp72) and inducible (Hsp69) forms co-migrated with and were immunologically similar to the Hsp isomers described for *C. gigas* and *O. edulis* (Fig. 3; Clegg et al. 1998, Piano et al. 2002). Oysters maintained in ambient seawater (15°C) and individuals that were assayed 60 min after heat shock (60 min at 34°C) were indistinguishable in Hsp70 profiles. Both possessed Hsp72 and Hsp77 (Fig. 3). By 24 h post-heat shock, however, the inducible isoform, Hsp69, as well as the two constitutive Hsp70s, were present (Fig. 3). The lowest heat-shock temperature at which Hsp69 was consistently induced (T_{on}) was 34°C regardless of season, whereas the highest heat-shock temperature (T_{off}) at which Hsp69 was consistently seen was 38°C (39°C was the lethal temperature), also regardless of season (Fig. 4). Hsp69 was maximal (assayed at 48 h post-heat shock) when the heat shock temperature was between 35 and 38°C during the summer and 32–38°C during the winter, but there was no significant difference in the level of Hsp69 expression for specific heat shock temperatures between seasons (Fig. 4). Likewise, the overall levels of the constitutive forms, Hsp72 and Hsp77, did not follow seasonal patterns, nor did they change significantly as a result of heat shock.

Once induced, Hsp69 remained present in adults for at least 3 days, but was maximal at the 24 and 48 h post-heat shock sampling times (Fig. 5). Quantities of Hsp69 were statistically indistinguishable at 24 and 48 h within all seasonal samples, and were significantly reduced by day 3 in two of the collections, summer, 2000 and winter, 2001. In all collections there was a significant decline in Hsp69 from 24–48 h post-heat shock levels to the 3–7 day levels ($P < 0.05$; Fig. 5). And, although not statistically significant, this declining trend was evident between 24 and 48 h and between 3 and 7 days in all collections (Fig. 5).

Long-Term Survival of Adults After Induced Thermotolerance

Heat shock did induce thermotolerance in *O. conchaphila* but only over a narrow time span. Initial experiments showed that tolerance to the naïve lethal temperature of 39°C was present at 24 h post-sublethal heat shock but not at 48 h. None of the oysters that were heat shocked at 34°C and then exposed to 39°C at 48 h post-heat shock survived a week, whereas 100% that were exposed to 39°C at 24 h post-heat shock, survived. Hsp69 levels did not reflect the decline in acquired thermal tolerance; at 24 and 48 h Hsp69 levels were not significantly different ($P > 0.05$; see Fig. 5). Only in one collection (summer 2000) was there a significant difference between Hsp69 levels at 3 days post-heat shock (Fig. 5). Because the duration of induced thermal tolerance was much shorter than that reported for other oysters (Clegg et al. 1998), we investigated whether long-term survival was compromised by post heat-shock exposure to lethal temperatures. Animals that were exposed to 38.5 or 39°C at 24 h after a 34°C sublethal heat-shock exhibited temperature dependent survival. Pre-heat shock animals that were placed directly from 12°C into lethal temperature sea-

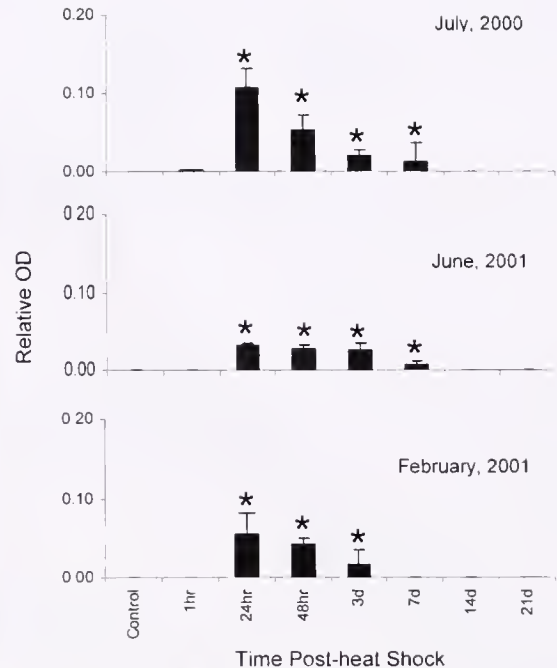


Figure 5. Hsp69 levels as a function of time (hours & days) post-heat shock. Relative levels of Hsp69 were assayed for up to 21 days after a 34°C heat shock of adult oysters collected during summer 2000, winter 2001, and summer 2001. Levels of post-heat shock Hsp69 were significantly different than 15°C controls ($*P < 0.05$) at 24 h, 48 h, and 3 days post-heat shock for all three collections. The only between season significant obtained was that Hsp69 was present through 7 days post-heat shock for the summer, but not winter collections.

water, 38.5 or 39°C, died also in a temperature dependent manner over the course of the experiment. All naïve adults that were exposed to 39°C died within 6 days while those exposed to 38.5°C perished over the course of 20 days, $36.7 \pm 19.6\%$ (SE) survived 10 days and $20.7 \pm 10.4\%$ survived to 20 days (Fig. 6A). In contrast, all of the adults that were heat shocked at 34°C prior to 38.5 or 39°C exposure at 24 h post-heat shock, survived 6 days (Fig. 6B). After 20 days $88.7 \pm 5.7\%$ of adults subjected to 38.5°C and $66.7 \pm 9.5\%$ of those exposed to 39°C survived, significantly higher survival than in the nonheat shock groups ($P < 0.05$, Fig. 6). Survival of the oysters exposed to the 38.5°C lethal temperature post-heat shock was significantly higher than that of the 39°C group after 20 days ($P < 0.05$).

Larval Heat Shock Response

All four developmental stages, early cleavage, late cleavage, blastula, and prerelease veliger larva, contained constitutive Hsp77 and Hsp72; however, only the veliger larval stage possessed Hsp69 after heat shock (Fig. 7). Hsp69 was present in veliger larvae by 6 h post-heat shock and had increased by a factor of four at 12 h post-heat shock (Fig. 8). In contrast to the temporal increase in Hsp69 over the first 12 h post-heat shock, thermotolerance decreased (Fig. 9). Heat shocked larvae were more tolerant to a 39°C post-heat shock treatment at 6 h after heat shock than at 12 h post-heat shock.

DISCUSSION

The preheat shock and post-heat shock thermal tolerance profiles of Hsp70 isoforms in *O. conchaphila* adults are similar to that

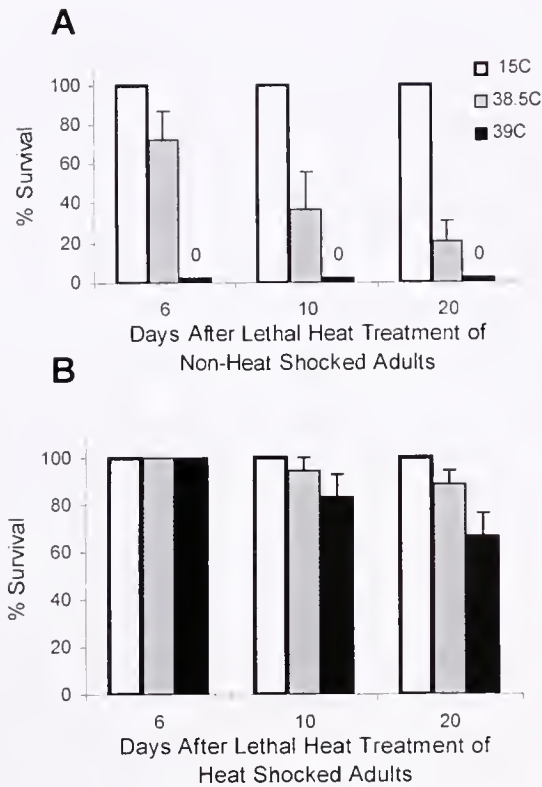


Figure 6. Long-term survival of adult oysters after sublethal heat shock followed by a lethal temperature exposure. **A.** Animals were heat shocked at the naïve lethal temperature of 38.5 or 39°C (ITT 38.5 and ITT 39). Survival was assessed at days 6, 10, and 20. Control animals were kept at ambient (15°C) temperature. **B.** Animals were heat shocked at the sublethal 34°C and after 24 h at ambient, heat shocked at a naïve lethal temperature of 38.5 or 39°C (ITT 38.5 and ITT 39). Control animals were kept in ambient (15°C) temperature following sublethal heat shock. Survival was assessed at days 6, 10, and 20 postlethal heat treatment ($n = 3$ replicates of 6 animals at each condition with each data point \pm SEM).

reported for a closely related oyster, *Ostrea edulis*, (see Pirano et al. 2002). Adults of *O. conchaphila* exhibited a pre-heat shock thermal limit between 38 and 39°C, whereas the thermal ceiling in *O. edulis*, was between 39 and 40°C (Pirano et al. 2002). Both species initiate expression of inducible Hsp69 at 32–33°C, and express Hsp69 maximally at 24–48 h post heat shock (Figs. 2 and 5; Pirano et al. 2002). In the present study we have shown that exposure of *O. conchaphila*, to a sublethal temperature high enough to induce Hsp69 expression also induces tolerance to the pre-heat shock lethal temperature of 39°C that lasts for 24 h. This induced thermal tolerance is not as dramatic as that seen in either *Crassostrea* or *Mytilus* species and, in part, explain the comparatively restricted geographical and bathymetric distribution of *O. conchaphila* (Hopkins 1937).

Research into the heat shock response of intertidal molluscs supports the concept of thermal plasticity. Both *Crassostrea* and *Tegula* modulate temperature tolerance ranges and thresholds of Hsp production based on acclimation to changes in environmental conditions such as seasonal temperature shifts or tidal height exposure (Roberts et al. 1997, Tomanek & Somero 1999, Buckley et al. 2001, Hamdoun et al. 2002). Hamdoun et al. (2003) demonstrated that seasonal increases in ambient temperature led to elevated levels of constitutive Hsps (Hsp77 and Hsp72), and to

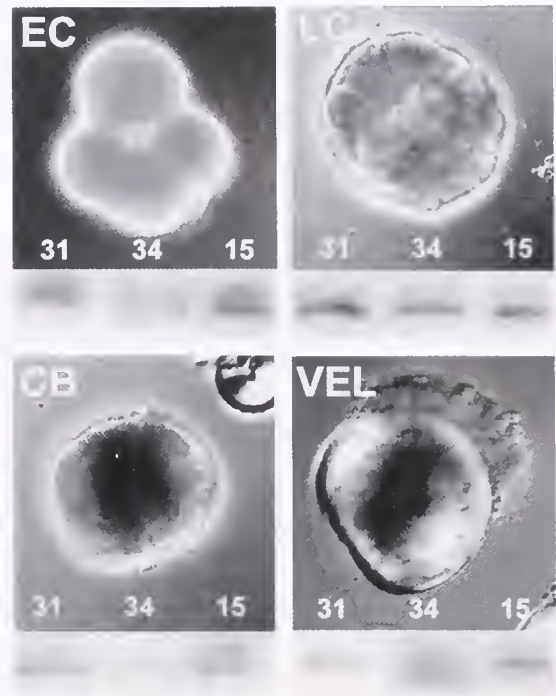


Figure 7. Hsp70 profiles of four different early life stages of *O. conchaphila*. EC, early cleavage; LC, late cleavage; CB, ciliated blastula; VEL, veliger larva. The four stages were heat shocked for 1 h at 31 or 34°C, after which samples were assayed for the presence of Hsp70 proteins. Only the veliger larva possessed Hsp69 and did so only after heat shock at 34°C.

seasonal increases (2–3 degrees) in pre-heat shock thermal tolerance. Thus, *C. gigas* is capable of adjusting its thermal response to accommodate baseline environmental conditions. *Ostreola conchaphila* did not exhibit a significant seasonal change in constitutive Hsp levels, nor was there a shift in Hsp69 T_{on} or change in the upper thermal tolerance limit although the mean summer temperature was twice that of the mean winter temperature (18.3 and 17.3°C vs 9.4°C). It appears that *O. conchaphila*, does not possess the capability of adapting to seasonal temperature shifts that is

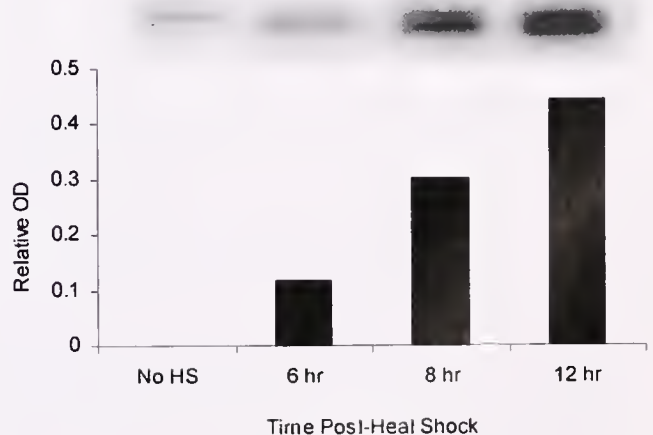


Figure 8. Detection of larval Hsp69 after heat shock. Veliger larvae were heat shocked at 34°C and assayed for the presence of Hsp69 at 6, 8, and 12 h post-heat shock.

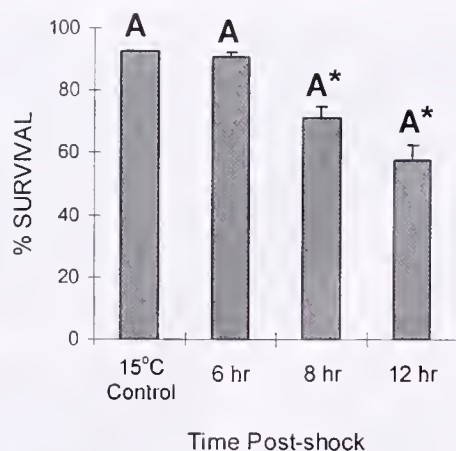


Figure 9. Duration of induced thermal tolerance in veliger larvae. Veliger larvae were heat shocked at 34°C and subsequently given a second thermal shock at 39°C (a lethal temperature to naïve larvae) at 6, 8, or 12 h post-heat shock. Mortality was assessed at 1 h later. Larvae at both 8 and 12 h post-heat shock (A*) had significantly higher mortality ($P < 0.05$) after a lethal heat shock than did non-heat shocked controls or 6 h post-shock larvae (A).

exhibited by other intertidal bivalves examined to date (Tomanek & Somero, 1999, Buckley et al. 2001, Hamdoun et al. 2003), reinforcing the idea that the distribution of *Ostrea* is primarily temperature determined.

Ostrea conchaphila are protandric hermaphrodites that exhibit a larviparous form of reproduction. Embryonic and early larval stages are brooded within the mantle cavity of female stage adults during the late spring and early summer, after which larvae are released as planktotrophic veligers (Santos 1992). Developmental stages are thus captive to temperature conditions experienced by brooding adults, temperatures that can approach adult lethal limits (Korringa 1976b). The fact that the lethal temperature for early life stages of *O. conchaphila* is the same as that for adults (38.5–39°C) may be an adaptation to survive captive (brooded) developmental periods. Typically early life stages are less tolerant of environmental stresses than is the adult life stage. Our results show that embryonic stages prior to gastrulation (early cleavage, late cleavage, ciliated blastula) were not able to mount a heat shock response, but

that the veliger larvae were able to do so. This developmental stage discrepancy to mount a heat shock response indicates that the embryonic genome is not transcriptionally active during early developmental stages and that maternal message for Hsp69 is not present as has been described for other species (e. g sea urchin; for a review see Giudice et al. 1999).

Interesting differences were observed between induced thermal tolerance and the presence of Hsp69 in veliger larvae that did not parallel tolerance and Hsp69 patterns seen in adults. While the quantity of Hsp69 in veliger larvae increased for the first 12 h post-heat shock, the ability to survive the naïve lethal temperature (39°C) after heat shock declined between 6 and 12 h. This could be explained by either a disconnect between the presence of Hsp69 and thermal tolerance in veliger larvae or by a general decline in condition, not necessarily heat shock associated, that lessens a larvae's ability to withstand stress. Larvae that are pre-maturely removed from brooding females, as was the case in the present study, typically do not survive to settlement (unpublished observations). Thus, young veliger larvae appear to require the environment of the mantle cavity; seawater is not sufficient for development until larvae have matured sufficient for natural release. Whether differences in the relationship between Hsp69 levels and thermal tolerance observed between adults and veliger larvae represent inherent life stage-specific physiologic states or are related to early removal of larvae from the mantle cavity is not known. To delineate which is operative, the heat shock response of brooded larvae for which the parent has been heat shocked prior to larval release needs to be investigated. Regardless of the duration of the heat shock response and relationship to Hsp69 levels, larvae that possess Hsp69 do produce a heat shock response that is within the same tolerance range as adults.

ACKNOWLEDGMENTS

The authors thank Karl Menard (Bodega Marine Lab) and Eric Larson (California Department of Fish and Game) for aid in obtaining adult *O. conchaphila*. The research was supported by the City of San Francisco. The authors also acknowledge the "Environmental Stress and Development in Marine Organisms" Summer Course at Bodega Marine Lab (ETX/NUT 127) for providing the support and the opportunity for A.M. Briden and T. Stokell to conduct portions of this research.

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CORRELATION OF ODOR AND COLOR PROFILES OF OYSTERS (*CRASSOSTREA VIRGINICA*) WITH ELECTRONIC NOSE AND COLOR MACHINE VISION

ÖZLEM TOKUŞOĞLU¹* AND MURAT Ö. BALABAN²

¹Ege University, Engineering Faculty, Department of Food Engineering, 35100, Bornova, Izmir, Turkey;

²University of Florida, Food Science and Human Nutrition Department, Gainesville, FL, USA 32611

ABSTRACT Odor and color changes in oysters (*Crassostrea virginica*) stored at 1.8° and 7°C were measured every 3 days for up to 13 days with an electronic nose, a computer vision system, and sensory panels. Electronic nose and odor sensory data was analyzed using Discriminant Function Analysis (DFA). Correct classification rates of 100% were obtained to group electronic nose data by days and sensory scores at each temperature. When all temperature and days data were pooled, DFA predicted sensory scores based on electronic nose readings with 94% accuracy. Oyster colors were analyzed using the 512 color block scheme. Eight colors were found to be significant. The correlations of these colors, as well as the average L*a*b* values with time and with color sensory data suggested prediction of color quality. This study suggests that electronic nose and color vision could be used to objectively evaluate the quality of oysters.

KEY WORDS: oyster, E-Nose, machine vision, odor and visual quality

INTRODUCTION

Oysters are a valuable commodity worldwide. In 1998, United States' domestic landings of oysters by weight of meat was 15,213 metric tons, valued at \$88 million (U.S. Department of Commerce, 1999). Imports increased from 4,506 metric tons in 1997 to 6,169 metric tons, valued at \$25 million, in 1998.

Oysters have a distinctive odor and flavor. Fresh shellfish products indicates typical aquatic fresh odor, brilliant appearance and physical peculiarities of the species in good situation (Perkins et al. 1992). The odor of seafood has been widely used as one of the signs of quality for a long time (Botta et al. 1995), the flesh color is a measure of the freshness of the seafood and research proves that consumers equate freshness with the vibrancy of the flesh color (Beaudoin, 1997).

Most studies regarding shellfish quality have focused on taste-active and flavor components (Sekiwa et al., 1997; Ok et al., 1996; Lee et al., 1995; Tanchotikul and Hsieh, 1991). Effects of chemical and other treatments on the taste and color of shellfish were also studied (Jawahar et al., 1994; Chellappan, 1991).

Fresh shellfish products should be marketed as fast as possible since their shelf life is very short. A critical commercial step after sorting is the inspection of the product. Current techniques concerning the quality assessments of oysters rely on sensory inspections that use the senses of vision, smell, etc. These are difficult to quantify to compare with objective standards and their results are not sufficiently repeatable. There is a necessity for objective, more reliable and quick technique to evaluate the quality of shucked oyster, to serve for these sector and international markets.

Developments in sensor technology and electronic noses (EN) have many potential applications in the food industry (Bartlett et al., 1997; Corcoran et al., 1993) including flavor and aroma profiles fresh squeezed orange juice, coffee powder, beer and cheese (Bazemore et al., 1996; Delaure et al., 1996; Pearce et al., 1993; Jou and Harper, 1998), quality control area of grapes, olive oil, ground beef, (Tokuşoğlu and Balaban, 2000; Aparicio et al., 2000; Winquist et al., 1993), aroma discrimination of black teas (Tokuşoğlu et al. 2002; Tokuşoğlu, 2001), separation of spice and grain mixture (Brezmes et al., 1997; Borjesson et al., 1996),

among others. Some work was published on the correlation of EN sensor outputs with sensory data in seafood products. EN has been applied to odor evaluation of seafood (Dodd et al., 2000; Luzuriaga, 1999; Balaban et al., 1996; Balaban et al., 1994).

Computer vision is being used in some food and agricultural areas to automate the inspection of visual quality (Sarkar et al., 1991). Visual inspection of seafood products involves image processing software and video cameras to evaluate product color, appearance, size or shape. There is reported research in the application of the machine vision technology for quality evaluation of seafood (Balaban et al., 1994; Balaban et al., 1996; Luzuriaga et al., 1995; Luzuriaga et al., 1997; Newman et al., 1998), for grading and sorting (Par et al., 1995; Diehl et al., 1990) and for shape detection (So et al., 1996). No study could be found concerning quality determination of oysters with E-nose and computer vision for objective evaluation. The objectives of this study were 1) to develop predictive models of odor change in shucked oysters stored at 1.8, and 7°C using an electronic nose, 2) to correlate these readings with sensory evaluation data, 3) to develop models of color change in these samples using machine vision and correlate them with visual sensory evaluation data.

MATERIALS AND METHODS

Oyster Samples and Storage Conditions

Fresh oysters (*Crassostrea virginica*) from Apalachicola, FL in the Gulf of Mexico were obtained. The weight of each oyster with the shell was approximately 75 g; the meat was approximately 20% of the total weight, about 15 g. Dating from the first day, they were stored in 100 ml beakers, 2 oysters per covered beaker, in cold rooms at 1.8, and 7.0°C for up to 13 days. For the machine vision experiments, 2 oysters were placed in a small petri dish (60 mm diameter), the lids covered, and stored at the same temperatures. The samples were analyzed every three days. This study was repeated twice.

Electronic Nose Measurements

An Electronic Nose model 4000 (EEV Inc, UK) equipped with 12 conducting polymer sensors (types 458, 459, 460, 461, 462,

*Corresponding author. E-mail: otokusoglu@superonline.com

463, 464, 478, 483, 297, 298, 401) was used. Before the experiment started, E-nose was calibrated with propylene glycol (chromatographic grade, Fischer Scientific, No. P-355-20, Fair Lawn, NJ; 75% v/v) as recommended by the manufacturer.

Every day before the experiments, compressed dry air (CGA Grade D, Strade Welding Supply Inc., Jacksonville, FL) was passed through the EN sensors for 20 min. A covered beaker containing oyster samples was taken out of the cold room one hour before analysis and equilibrated to room temperature (25 °C). The beaker was then placed in the EN. Five consecutive readings were taken for each sample. Then, the replicate sample was taken out of the cold room and treated similarly. For each reading, the vessel and head were purged with dry air for 2 and 4 min, respectively. Then, sensor responses were read for 4 min. For each sample total analysis time was 10 min.

Computer Vision Analysis

The color machine vision system developed by Luzuriaga and Balaban (1997) was used to grab images. The light box had 3 sections consisting of a top lighting box, a bottom lighting box and a sample chamber. The illuminant was 15 watt Chroma 50 fluorescent light (F15T8-C50, General Electric, Cleveland, OH). The S-video output of a video camera (Sony SSC-S20, Sony Corp., Japan) was connected to a color frame grabber (Meteor, Matrox Electronic Systems, Ltd., Dorval, Quebec) mounted inside a Pentium 133 MHz PC computer. In this experiment, the settings for the video camera were: hue = 0, saturation = 160, contrast = 160, and brightness = 148 (for a range from 0 to 255). During image acquisition a standard color tile (beige, L = 63.59, a = 65.19, b = 33.39) was placed next to the oyster samples in the light box.

For each sampling day, the small petri dish was removed from the cold room, placed in the light box with front and back lighting, the image of oysters stored 1.8 and 7.0 °C was captured and saved in a computer file. The petri dish was then placed back in the cold room. The same samples were used throughout the experiment. This study was repeated twice. Oysters remained at room temperature for no more than about 5 min during image capture.

Images were calibrated by the color analysis software using the standard color tile (Luzuriaga, 1999). The color of the calibrated images was reported in the RGB and L, a, b color systems. The 512-color blocks scheme was used for the RGB colors (Luzuriaga, 1999). The value for each color block was the percent of the view area of the oyster covered by that color. Colors that were more than 5% of the total area were considered.

Sensory Evaluation

The odor and color of oyster samples were analyzed by a 10-member trained sensory panel consisting of graduate students, professors, and personal, 24-50 y of age, from the Food Science and Human Nutrition Dept. at the University of Florida. Panelists were trained using oyster samples from the same supplier utilizing a descriptive sensory analysis method. They used the samples as "excellent" and "poor" as a guide in their decision to evaluate odor and color for the unknown samples, respectively. Samples stored at two different temperatures were evaluated using odor description for the best sample; typical aquatic fresh odor, distinctive oyster aroma, and the worst sample; deteriorated odor, putrefactive aroma. For color description the best sample had brilliant color,

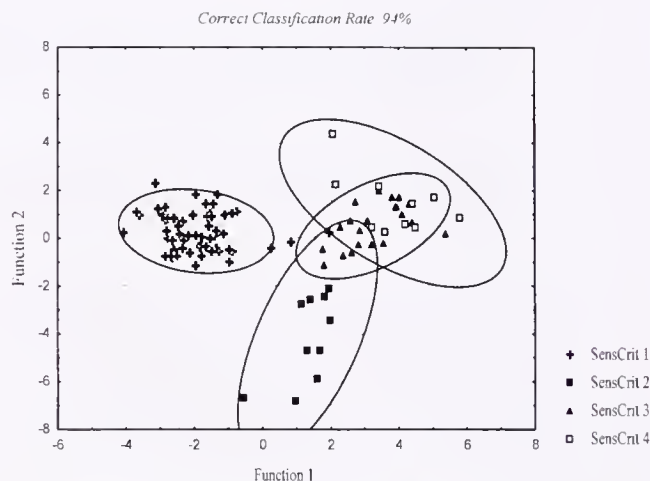


Figure 1. DFA of oyster odor based on sensory criteria from both temperatures pooled together and EN readings (Ellipses = 90% confidence area).

good appearance, and the worst sample had deteriorated, dull, slimy appearance.

A 5-point scale was used in this study where 1 = poor, 2 = fair, 3 = good, 4 = very good, 5 = excellent. Samples were evaluated for odor and color every three day for up to 13 days. This study was applied according to IFT Sensory Evaluation Division (IFT, 1994). Odor and color profile in descriptive sensory profiling was applied as a method (Piggott et al., 1998) and hedonic scale was carried out as an overall acceptability test in consumer and acceptance tests.

Data Analysis

Discriminant Function Analysis (DFA), a parametric procedure to identify relationships between qualitative variables such as sensory descriptors, odor, color classes, and quantitative variables such as sensor response, was used. It tries to classify samples into known groups by constructing linear relationships for the sensor data or color data and the criterion set of variables. This method generates functions that are used to classify samples. Each discriminant function is calculated for which the F-ratio of the analysis of the variance is maximized (Gardner and Hines, 1997).

$$\text{Function } F_i = \sum_{j=1}^{12} a_{ij} x_{ij} + a_i \quad (1)$$

Function 1 gives the most separation between groups, function

TABLE 1.
Percent Correct Classification Rates for the DFA of EN Readings Compared with Sensory Scores.

Sensory Score	1.8 °C	7.0 °C	Combined T
	(n = 10)	(n = 10)	(n = 20)
1	100	100	97
2	—	100	100
3	100	100	100
4	100	—	60
Overall	100	100	94

n = number of EN readings used to obtain the discriminant functions.

TABLE 2.
Odor Sensory Scores Obtained by Sensory Panel.^a

ODOR (DAY)	Sensory scores	Sensory scores
	1.8°C	7.0°C
	Average	Average
0	5	5
1	4	3
3	3	2
6	1	1
9	1	1
13	1	1

^a ($p < 0.01$).

2 gives the next best separation, and so on. Generally the first two functions are sufficient to separate the groups. E-nose sensor readings, color vision data and sensory scores were analyzed in Statistica for Windows ('98 edition, Ver. 6.0, StatSoft Inc., Tulsa, OK) using discriminant function analysis (DFA) to develop the predictive functions for oysters stored at different temperatures and storage times, and to correlate these with sensory data.

In this study, the 12 sensor outputs in E-nose analysis and 8 color outputs obtained from color analysis were reduced to 2 discriminant functions. These functions were used to graph the data in two dimensional plots and observe separation of groups. For each function, coefficients and correct classification matrix were obtained.

RESULTS AND DISCUSSION

E-nose readings with odor sensory evaluation data for oyster stored at different temperatures for different times were correlated and also machine vision readings with color sensory evaluations for oyster stored at two different temperature for up to 13 days were correlated using Discriminant Function Analysis. Figure 1 indicates DFA correlations of EN readings with sensory criteria data at two storage temperature. For 1.8 and 7.0°C, all temperature

TABLE 3.
Coefficients for DFA of Oyster Odor Based on Storage Times and EN Readings.

Sensor Type	Coefficients for Discriminant Functions					
	Storage at 1.8°C		Storage at 7.0°C		Both temperatures	
	Funct. 1	Funct. 2	Funct. 1	Funct. 2	Funct. 1	Funct. 2
Type 301	-1.90	10.70	-3.26	4.06	-5.06	4.71
Type 298	-14.77	37.13	3.49	8.69	-26.91	31.05
Type 297	47.72	-33.80	-5.76	-4.65	39.38	-38.07
Type 283	1.35	-14.18	3.54	-13.23	12.21	-5.75
Type 278	-12.55	-17.17	0.74	-6.48	0.69	-3.87
Type 264	-3.21	65.60	-7.01	17.08	-29.68	31.96
Type 263	-7.78	-26.87	22.52	-3.44	11.03	-2.82
Type 262	14.40	-4.97	-4.05	3.58	6.74	-10.92
Type 261	-3.78	-4.86	-13.25	-11.67	-8.20	-14.83
Type 260	18.71	18.76	-0.20	-0.25	9.76	13.22
Type 259	-9.43	-30.43	9.38	5.52	9.44	-8.57
Type 258	-29.02	0.19	-6.23	0.90	-19.00	3.84

TABLE 4.
Percent Correct Classification Rates for the DFA of EN Readings Compared with Storage Time.

(DAY)	1.8°C	7.0°C	Combined T
	(n = 10)	(n = 10)	(n = 20)
1	100	100	100
3	100	100	100
6	100	100	95
9	100	100	100
13	100	100	95

n = number of EN readings used to obtain the discriminant functions.

and days pooled data, discriminant function analysis perfectly separated the E-nose data with correct classification rates of 100%, 100%, 94% respectively (Table 1).

There was no significant difference between the number of EN readings (n) used to obtain the discriminant functions ($P < 0.01$).

During sensory panel, panelists judged the differences in odors at two different temperature for up to 13 days (Table 2). Table 2 indicates odor sensory scores of oysters stored at 1.8 and 7.0°C by

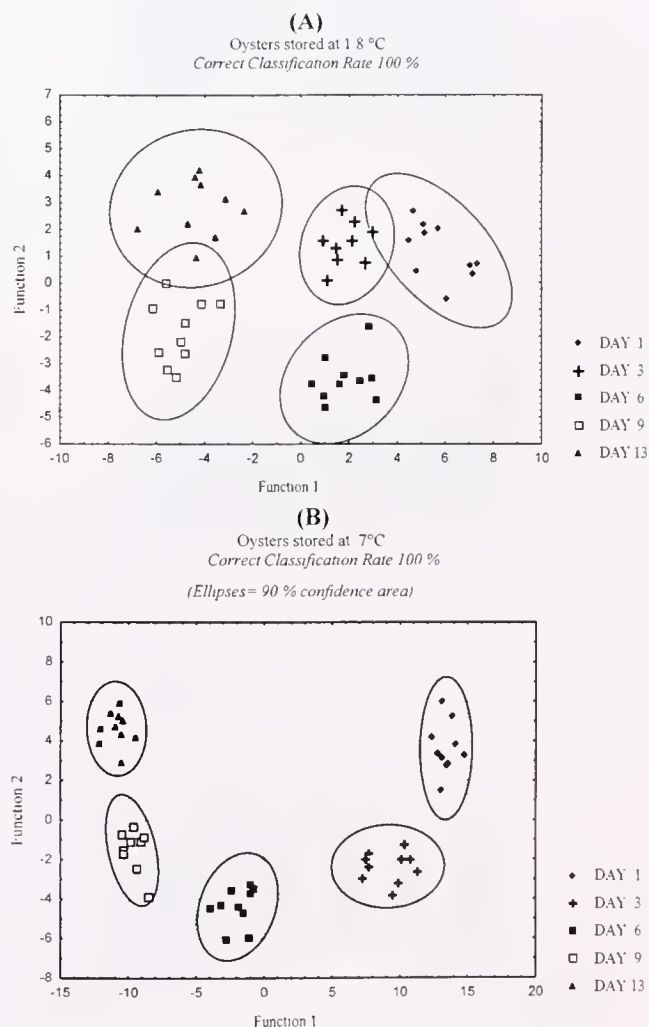


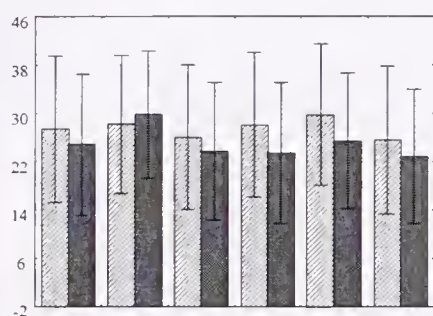
Figure 2. DFA of oyster odor based on storage time and EN readings.

sensory panel. Oyster odor changed with storage time. The differences between the two replicate of sensory scores of both temperatures were not significant ($P < 0.01$).

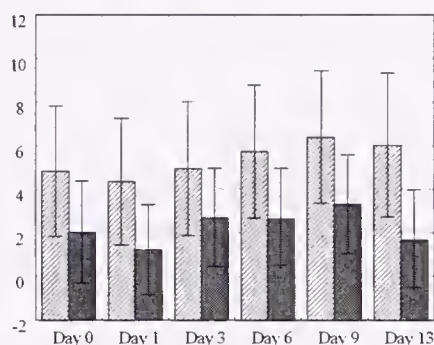
At two temperatures, some panelists could evaluate odor differences with difficulty between successive days while EN detected these differences clearly. After Day 3, panelists could not discriminate odor of semideteriorated and deteriorated oyster samples and these were evaluated as putrefactive aroma. When all temperature and days data were pooled together, DFA predicted sensory scores based on electronic nose readings with 94% accuracy.

DFA was used to calculate two discriminant functions to correlate EN readings with storage time at both temperature. Table 3 shows coefficients for Discriminant Functions to estimate oyster odor based on storage times at 1.8°C, 7.0°C and both temperatures with EN.

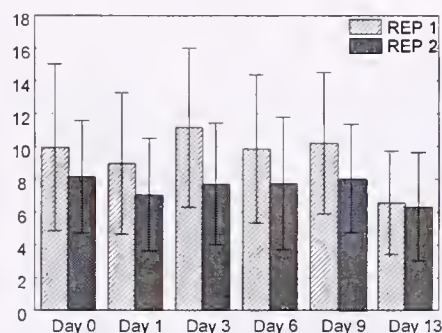
DFA analysis of e-nose odor data correlated with storage time



Average L values of all pixels, both replicates shown



Average a values of all pixels, both replicates shown



Average b values of all pixels, both replicates shown

Figure 3. L*a*b* values, oysters stored at 1.8°C (± 1 SD) shown.

at 1.8, 7.0°C gave correct classification rates of 100% and 100%, respectively (Table 4). There was no differences between the number of Electronic Nose readings (n) used to obtain the discriminant functions ($P < 0.01$). Figure 2 shows a clear discrimination for up to 13 days at both temperature. As shown in these figures, there was no overlap in days, meaning that there were definite differences in the sensor readings for each day of storage.

Computer vision could clearly quantify non-uniform colors of oyster. Classification functions of the correlation between color data and storage time, as well as color sensory evaluation data, were obtained. When classified by day at each temperature, the classification rates for 1.8°C and 7.0°C were 100% and 100%, respectively. Classification of color data by sensory readings gave classification rate of 100%, 100%, respectively.

Eight colors were found to be significant for oyster color quality with RGB color system. The correlations of these colors, as well as the average L*a*b* values with time and with color sensory data suggested prediction of color quality.

Figure 3 shows average L*a*b* values of oysters stored at 1.8°C. The lightness scale (L) differences of oyster samples for both replicates were not significant ($p < 0.01$). Redness or greenness (a) and yellowness or blueness (b) can reflect a single color function called color difference, and this difference is a measure of the distance in color space between two colors. According to L*a*b* results, the differences of oyster color stored for up to 13 days could not be clearly determined (Fig. 3).

The panelists judged differences in color quality in both temperatures during storage time and in the same samples evaluated for up to 13 days (Table 5). Table 5 indicates color sensory scores of oysters stored at 1.8°C and 7°C by the sensory panel. Oyster color changed with storage time. The differences between the two replicates of sensory scores of both temperatures were not significant ($p < 0.01$).

At 1.8°C and 7.0°C, panelists could not evaluate color quality differences after day 3, whereas machine vision clearly determined these differences between semideteriorated and dull, slimy samples.

Each color block of oyster and RGB values of these colors were determined using the color machine vision system and the eight colors obtained are shown in Table 6.

Six of the 8 colors were identified as deteriorated color whereas the remaining two were represented by yellow and orange hues.

During storage time, good color, or Color 73 (RGB = 245,241,273), was decreased over time, and fresh oyster appearance was decreased as show in Fig. 4(a). The level of this color

TABLE 5.
Color Sensory Scores Obtained by Sensory Panel.^a

COLOR (DAY)	Sensory scores	
	1.8°C	7.0°C
	Average	Average
0	5	5
1	4	3
3	3	2
6	1	1
9	1	1
13	1	1

^a ($p < 0.01$).

TABLE 6.
RGB Values of Each Color Block of Oyster.

COLOR NO	R value	G value	B value	COLOR
72	48	48	16	Dark olive
73	245	241	173	Pale orange yellow
137	67	107	68	Dark yellowish green
145	80	80	48	Greyish olive
146	80	80	80	Dark grey
209	112	80	48	Moderate brown
210	112	80	80	Dark red
218	112	112	80	Greyish olive

was decreased to around 17% for storage at 1.8°C and 8% for 7.0°C from around 29%. [Fig. 4(a)]. There were no significant differences between the two replicates of color analysis ($p < 0.01$). While computer vision was determined deterioration from after day 3, objectively; sensory criteria could not evaluate visual quality of oysters. Color (145 + 146) (RGB = 80,80,64) increased over time and level of this color was increased to around 27% for storage at 1.8°C and 36% for 7.0°C from around 16%, respectively [Fig. 4(b)]. These additional colors were responsible for the grayish-dark hue of deteriorated oyster.

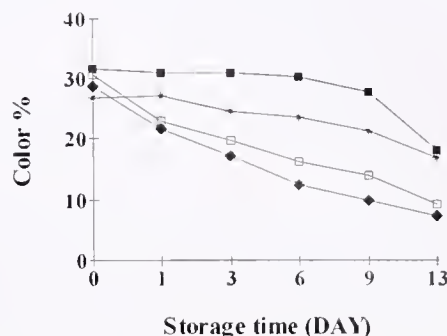
E-nose and machine vision data can be used in pattern recognition for objective classification of oyster. With comprehensive study oyster samples by odor and color, the e-nose can be used reliably to classify and machine vision can objectively classify oyster samples by color.

DFA can be used satisfactorily as a pattern recognition analysis technique in food engineering area for the identification and determination of seafood odor and color.

ACKNOWLEDGMENT

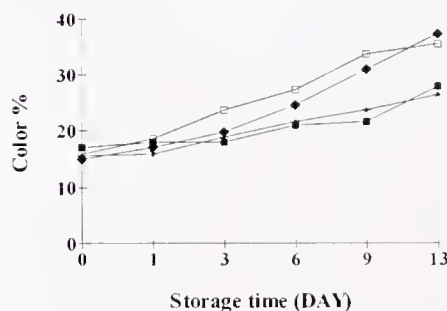
This research was supported in part by the Florida SeaGrant Project R/LR-Q-17 The part of this work was presented at the

Color No: 73 RGB=245,241,273



A

Color No: (145+146) RGB=80,80,64



B

Figure 4. Changes in the colors No: 73 (A) and No: (145+146) (B) over time.

"2002 IFT Annual Meeting June 15–19 in Anaheim Convention Center, Anaheim, California, USA."

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INTRASPECIFIC LIFE HISTORY VARIATION IN THE SOUTHERN OYSTER DRILL, *STRAMONITA HAEMASTOMA*: PATTERNS AND CAUSES

KENNETH M. BROWN,*¹ MICHAEL MCDONOUGH AND TERRY D. RICHARDSON

Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803;

Department of Biology, University of North Alabama, Florence, Alabama 35632

ABSTRACT Along the Louisiana coast of the Gulf of Mexico, the southern oyster drill, *Stramonita haemastoma* (Gray), reached peak densities at an exposed, coastal location, with lower but more constant densities in an estuarine oyster reef. Average oyster drill size in field samples was largest at the estuarine, intertidal reef and smallest at the exposed location. Caged oyster drills grew more rapidly and were more fecund at the subtidal, estuarine oyster reef than at the exposed location, resulting in higher biomass and production estimates. Life histories thus changed from slower growth, and delayed and semelparous reproduction at the exposed location to rapid growth, and earlier and iteroparous reproduction at the subtidal, estuarine oyster reef. When oysters were added to cages at the exposed location (where they are rare), oyster drill growth and reproductive rates increased dramatically. Trapping revealed no obvious differences in the abundances of crab predators between the estuarine and exposed site, nor were there any differences in gastropod shell thickness (e.g., vulnerability to predators). We conclude that the availability of profitable prey like oysters plays a greater role in explaining oyster drill life history variation than predation risk, and that wave exposure has only indirect effects, by limiting the availability of oysters or forcing oyster drills to feed on smaller prey.

KEY WORDS: environmental causes, life history, oyster drill, population ecology, *Stramonita haemastoma*

INTRODUCTION

The southern oyster drill, *Stramonita* (= *Thais*, Kool 1987) *haemastoma* Gray, an important predator of oysters (Brown & Richardson 1987), is common throughout the Gulf of Mexico (Butler 1985). Along with the black drum (*Pogonias cromis*), it restricts the distributions of oysters in the Gulf of Mexico to estuarine areas where salinities are too low for most predators (Chantry et al. 1983, Wilber 1992), or to the high intertidal at coastal locations (Bahr & Lanier 1981, Brown 1997, Brown & Stickle 2002, Roegner & Mann 1995). Recent research on oyster drills has concentrated on tolerance and capacity adaptations to salinity, temperature, and hypoxia (Brown & Stickle 2002, Stickle 1985, Stickle 1999), population structure (Liu et al. 1991), and foraging behavior (Brown 1997, Brown & Alexander 1994, Brown & Richardson 1987, Richardson & Brown 1990, Richardson & Brown 1992). Relatively little is known, however, about intraspecific variation in oyster drill life history characters such as growth or fecundity.

This study documents variation in density, growth, and reproduction between oyster drill populations in coastal and estuarine locations, and at a smaller scale, between an intertidal and a nearby subtidal oyster reef at the estuarine location. Contrasting coastal and estuarine sites is important because the lowered levels of wave activity (Richardson & Brown 1990) and salinity (Stickle 1985) at estuarine sites alter oyster drill feeding rates. Comparison of intertidal and subtidal sites is important because aerial exposure decreases oyster drill feeding rates (Brown & Stickle 2002). Specifically, we quantitatively sampled and measured oyster drills for a year at three locations (one coastal and two estuarine) and tagged snails at each location and caged them to follow growth in two seasons and reproductive activity during the egg-laying season in 1 yr. To integrate these life history data, we calculated rates of secondary production at each site, which combines in a single measure life history parameters like density, biomass, individual growth, development time, recruitment, and mortality (Benke 1993, Taylor 1998).

We also discuss what environmental factors may cause the variation, and provide information on two possible causes, food availability and predation risk. Prey abundance and quality have been shown to affect the ecology of thaidid snails on other coastlines (Palmer 1983, Spight 1982). The degree of predation risk can reduce feeding rates (Richardson & Brown 1992), lower growth rates, and result in increased shell thickness as a defensive mechanism (Palmer 1990). To evaluate the relative importance of prey availability and predation risk for *Stramonita haemastoma*, we (1) added oyster prey at a site where only barnacle prey were available, and (2) assessed predation risk by estimating the abundance of the stone crab (*Menippe adina*) and looked for differences among oyster drill populations in shell thickness.

MATERIALS AND METHODS

Site Descriptions

The three locations [for a map of locations, see Brown and Swearingen (1998)] are approximately 140 km south of New Orleans (29°10'N, 90°05'W). Caminada Pass (the exposed site, = CP) is the narrow mouth of Barataria and Caminada Bays, an extensive estuary system southeast of New Orleans. Strong currents occur during tidal flows, and CP is fairly exposed to wave action from the Gulf. Using plaster casts, Richardson and Brown (1990) found that over three-quarters of the mass was lost over a 2-day interval at this site (Table 1), indicating much greater levels of exposure and flow than at the estuarine site, where only one-fifth of the mass was lost on average. Average temperatures vary from 12°C during winter months to 30°C during summer months (Fig. 1), and salinities average 26 psu (practical salinity scale, Table 1). By far the most common prey at this site are barnacles (*Balanus eburneus*) (Table 1).

The other two locations, near the Louisiana Universities' Marine Consortium laboratory at Port Fourchon, Louisiana, about 20 km west of Caminada Pass and 10 km inland from the mouth of Bayou Fourchon, are more estuarine (Table 1). The first location is an intertidal oyster reef (= Laboratory Int) in a *Spartina alterniflora* marsh, and the second site is a subtidal reef (= Laboratory

*Corresponding author. E-mail: kmbrown@lsu.edu

TABLE 1.

Comparison of two locations along the Louisiana coast as to salinity (psu, range), degree of wave exposure (percent of mass loss from plaster casts), and abundances and sizes of prey items of oyster drills.

Location	Salinity	Exposure	Prey	Oysters	Barnacles
Fourchon Lab	22 (16–28)	21%	Numbers/M ²	222 ± 9.5	13,885 ± 1,273
			Size	79.2 ± 56.7 g	11.0 ± 3.0 mm ²
Caminada Pass	26 (18–34)	79%	Numbers/M ²	4.0 ± 0.3	65,215 ± 8,310
			Size	10.6 ± 3.1 g	9.0 ± 1.2 mm ²

Standard errors are given for prey abundances and sizes. Oyster sizes refer to total wet mass (including shell). Barnacle sizes are basal areas. Data on prey abundance taken from Brown (1997), Brown and Swearingen (1998), McCoy and Brown (1998), and Banks and Brown (2002).

Sub) 50 m from the intertidal reef. Both sites are heavily colonized by oysters, with densities over a 5-yr period (pooled over both tidal heights) 50 times greater than at the exposed site (Table 1). Barnacle density, in contrast, is greater at the exposed site. The data in Table 1 were collected over a 5-yr period using quadrat sampling for oysters and colonization plates for barnacles (for more details, see papers given in Table 1).

Sampling

We quantitatively sampled all three locations (Table 2) on a monthly basis from August 1998 to October 2000 by haphazardly placing out 10–16 m² quadrats during low tide. Each quadrat was first searched visually to remove larger oyster drills, and oyster shell was then rinsed over a sieve to locate smaller drills (although we undoubtedly missed small, recently recruited individuals). All snails were measured to the nearest 0.1 mm. A two-way analysis of variance (ANOVA) was used to contrast densities and shell sizes among locations and through time. Locations were considered a fixed effect because they were selected to contrast different levels of exposure or tidal height, and dates were considered fixed effects as they were selected so that each month was represented to allow calculation of monthly production estimates. Density data were log-transformed to improve normality.

To assess differences in predation risk among sites we (1)

trapped crabs at Caminada Pass and the subtidal, estuarine reef, and (2) assessed differences in shell lip thickness of oyster drills at both sites. We fished six, baited crab traps at each site for two nights, on three dates (summer through fall 2001) and pooled all crabs collected per date at each location. Crab catch per unit effort (CPUE) data were log-transformed and subjected to a two-way ANOVA (two crab species × two locations with the three dates as replicates), and data were also analyzed in a nonparametric analog of the two-way ANOVA, the S. R. H. modification of the Kruskal-Wallis test (Sokal and Rohlf 1995).

Secondary Production

To estimate biomass from shell length, 100 snails from Caminada Pass and the subtidal, estuarine oyster reef were measured to the nearest mm, and tissue was dried at 60°C for 24 h. Dry tissue mass was estimated using the following equation ($R^2 = 0.95$, $P < 0.0001$):

$$\text{g dry mass} = (1.8 \times 10^{-6})(\text{shell length})^{3.46}.$$

Monthly, size-specific production (g dry tissue mass/m²) at each site was estimated using the instantaneous growth method (Benke 1996, Waters 1977). The size-specific instantaneous growth rate was estimated from the growth of similar-sized snails in experiments, and monthly, size-specific biomass estimates (g dry tissue mass/m²) from field samples. For each time interval, growth was averaged for all individuals within each millimeter size class and applied to size-specific, mean monthly biomass estimates. Production was calculated for each millimeter size class, then summed to estimate monthly production.

Growth Experiments

Two experiments were conducted from December 1998 to April 1999 and from June 1999 to August 1999 (Table 2) to assess seasonal differences in growth rates. Snails from Caminada Pass and the subtidal, estuarine oyster reef (but not the intertidal reef because of low snail abundance, see "Results") were mixed to produce a size range, and 10 individuals in each of four different size categories (averaging 30, 40, 50, and 60 mm mean shell length ± 1 mm SE) were placed separately in cages at all three locations. Cages were plastic trays (60 cm × 75 cm × 15 cm high) covered with 3-mm vexar mesh, and placed on the bottom and held fast by cable ties to PVC poles embedded 1 m in the sediment at all four corners. There were four replicate cages for each size class at each location. Snails were tagged with numbered bee tags attached with super glue and were measured (tip of spire to tip of aperture) to the nearest 0.1 mm with a Vernier caliper and weighed to the nearest

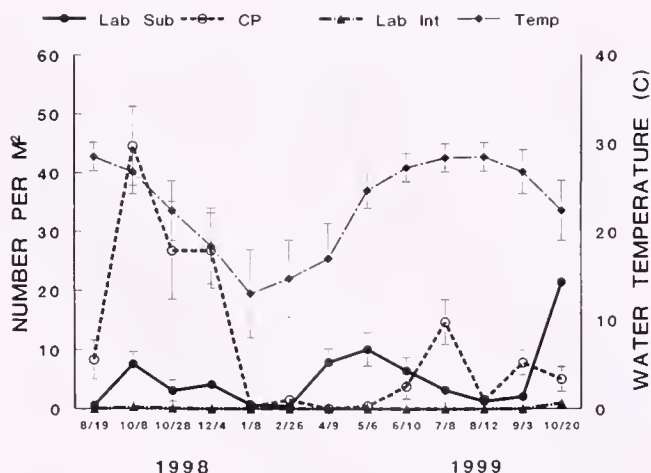


Figure 1. Variation through time in densities ($\bar{x} \pm \text{SE}$) of the southern oyster drill *Stramonita haemastoma* at three locations along the Louisiana Gulf coast. CP = Caminada Pass, Laboratory Sub = subtidal oyster reef at Port Fourchon, Laboratory Int = intertidal oyster reef at Port Fourchon. Water temperatures are monthly averages ($\pm \text{SE}$) from the NOAA Grand Isle buoy (located at Caminada Pass).

TABLE 2.

Summary of what sampling and experiments were conducted at each of the three locations, along with the types and periodicities of data collected.

Location	Field Sampling	Growth Experiments	Reproduction Experiment	Prey Addition Experiment
Lab Int.	X	X		
Lab Sub.	X	X	X	
Caminada Pass (=CP)	X	X	X	X
Data	Density, size distribution, production (g dry mass/M ²)	Individual growth (mm)	Numbers of egg capsules, numbers of embryos per capsule	Individual shell growth (mm) and capsules per cage
Periodicity and length	Monthly for 1 yr	Monthly for 3 mo	Monthly for 2 mo	Monthly for 2 mo

milligram with an analytical balance at the start and monthly through the end of the experiment.

The most common prey at Caminada Pass were barnacles that rapidly recruit to subtidal surfaces (see discussion above), and we simply allowed prey to colonize cages. At the two estuarine locations, we added 20 oysters (from 50 to 150 g wet total mass per oyster) to each cage to approximate the abundance and size range of oysters available at the locations (Brown 1997). As cages were checked monthly, oysters were added to replace those consumed (at the estuarine locations), and the mesh was cleaned to facilitate water circulation.

Gastropod growth rates are dependent on size, and we used Ford-Walford plots (Fig. 2) to illustrate how shell growth increments were related to initial shell length (Etter 1989). Plotted data are the total shell growth increment over the experiment versus initial size for each snail surviving the experiment. However, because 10 snails were placed in each cage, using individual snails as replicates in statistical analyses could be considered pseudoreplication. We therefore also used the average shell length per cage as the experimental unit and performed a repeated measures ANOVA to test for time effects (e.g., change in size since oyster drills were measured monthly), as well as the location effect and the location times time interaction (Proc GLM, Statistical Package (SAS) Inc., 1988).

Reproduction Experiments

These experiments (Table 2) were conducted in April 1999 through May 1999, during the peak of the oyster drill egg-laying season, at Caminada Pass and the subtidal, estuarine oyster reef (experiments were not replicated at the intertidal site because of low ambient snail densities). The same four size classes, with three replicate cages for each size class, each with 10 oyster drills, were deployed at each location in the same fashion as in the growth experiments. Cages were checked monthly, and all egg capsules removed. A randomly selected subset of five capsules from each cage was preserved in 70% ethanol and counted at 7× magnification to estimate embryo number. Southern oyster drill eggs hatch in approximately 2 wk (Roller & Stickle 1988), but capsules continue to adhere to surfaces, so monthly counts were not underestimates. Preliminary data indicated counting the embryos in five egg cases gave an accurate estimate of the average number of embryos per capsule. We used two-way ANOVAs to test for significant differences among locations and oyster drill shell lengths in number of capsules produced and average number of embryos per capsule. Data were checked for normality with procedure.

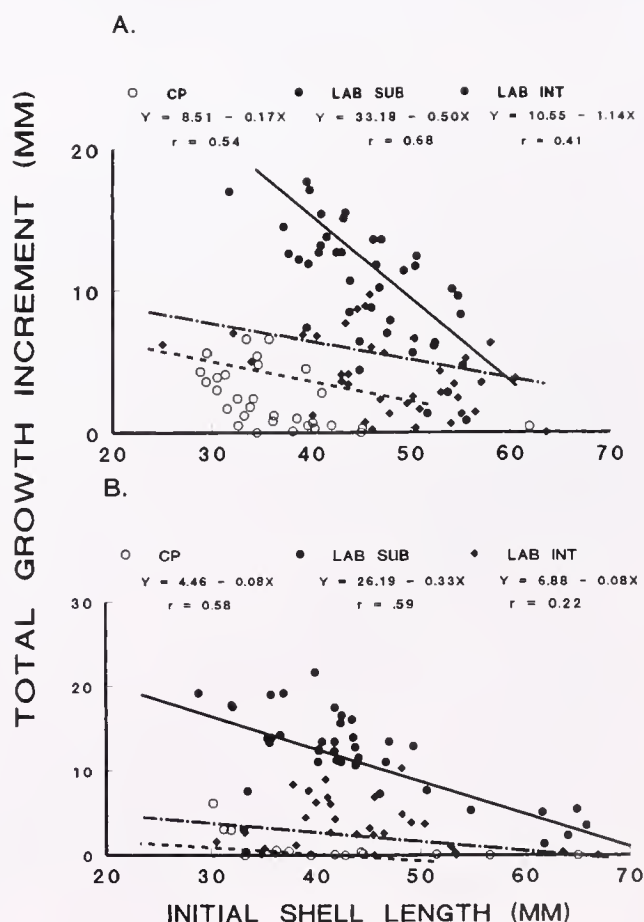


Figure 2. (A) Ford-Walford plot of total shell growth increment against initial shell length for oyster drills caged at three locations in the winter 1998-1999 growth experiment, along with fit regression lines and correlation coefficients. CP = Caminada Pass, Laboratory Sub = subtidal oyster reef at Port Fourchon, Laboratory Int = intertidal oyster reef at Port Fourchon. (B) Ford-Walford plot of shell growth increment against initial shell length for oyster drills caged at three locations in the summer 1999 growth experiment along with fit regression lines and correlation coefficients. CP = Caminada Pass, Laboratory Sub = subtidal oyster reef at Port Fourchon, Laboratory Int = intertidal oyster reef at Port Fourchon.

Univariate (SAS Institute 1988) but transformation was not necessary. Tukey's *a posteriori* tests were used to determine which means differed.

Prey Addition Experiment

To determine if reduced growth and fecundity at Caminada Pass were explained by the absence of high-quality prey, we added 20 oysters (wet total mass from 50 to 150 g per oyster) to cages and compared growth and fecundity to control animals in cages with "ambient" prey. On March 28, 2000, six control and six experimental cages were placed at Caminada Pass at the same site and in the same fashion as in earlier experiments. Each cage had 10 marked oyster drills (mean shell length \pm SE, control = 44.8 ± 1.2 , experimental = 45.9 ± 1.2) and was retrieved after 2 mo. Two control cages were damaged, but increments in average shell length and total number of capsules produced were contrasted between the four remaining control and six experimental cages in a one-way ANOVA.

RESULTS

Density, Size, and Secondary Production

Oyster drill densities varied significantly among the exposed and two estuarine locations ($F = 130.0$, $P < 0.0001$), months ($F = 22.3$, $P < 0.0001$), and a significant interaction occurred between location and month ($F = 15.7$, $P < 0.0001$). Overall, densities were highest at Caminada Pass, intermediate at the subtidal, estuarine oyster reef, and lowest at the intertidal oyster reef (Table 3). However, densities fluctuated considerably through time (Fig. 1), explaining the significant interactions between location and month.

There were also significant differences among the three locations ($F = 365.3$, $P < 0.0001$) and through time ($F = 160.1$, $P < 0.0001$) for average shell length as well as a significant interaction ($F = 127.2$, $P < 0.0001$). Tukey's *a posteriori* tests indicated oyster drills were largest at the intertidal estuarine oyster reef (Fig. 3, Table 3), intermediate in size at the subtidal, estuarine oyster reef, and smallest at Caminada Pass.

Unlike density, average biomass was higher at the subtidal estuarine oyster reef than at Caminada Pass (Table 3), reflecting the greater number of large snails at the estuarine, subtidal location. Mean monthly biomass at the intertidal reef location was much lower. Peak biomass among all locations was observed in April 1999 at the subtidal oyster reef, when biomass exceeded 20 g/m^2 . The higher biomass and markedly greater individual growth rates at the subtidal, estuarine oyster reef (see next section) resulted in a 10-fold higher average monthly production in comparison to Caminada Pass (Table 3).

TABLE 3.

Average oyster drill density per M^2 , shell length (mm), biomass (g per M^2), and production (g per M^2), \pm SE ($n = 12$ mo) at three locations along the Louisiana Gulf of Mexico coast.

Location	Density	Size	Biomass	Production
Lab Sub.	6.25 ± 2.52	49.6 ± 1.0	8.01 ± 1.36	1.46 ± 0.20
Lab Int.	0.04 ± 0.02	77.2 ± 2.7	0.74 ± 0.49	0.02 ± 0.01
Caminada Pass	11.40 ± 4.10	34.6 ± 2.0	4.04 ± 0.85	0.14 ± 0.03

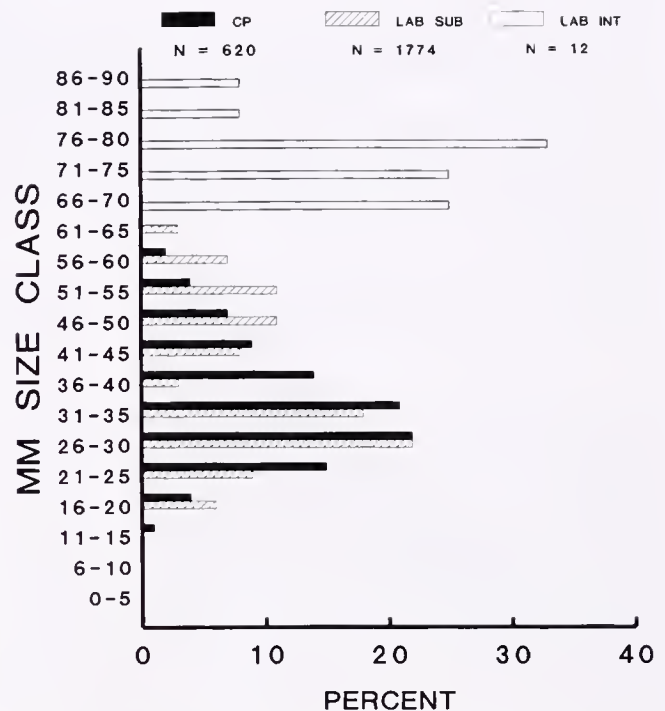


Figure 3. Size distributions of all southern oyster drills collected during sampling at the three locations along the Louisiana Gulf coast. CP = Caminada Pass, Laboratory Sub = subtidal oyster reef at Port Fourchon, Laboratory Int = intertidal oyster reef at Port Fourchon.

Predation Risk

Crab CPUE values differed among the two crab species, with blue crabs much more abundant than stone crabs (Table 4). The species effect was significant ($F = 20.0$, $P < 0.001$), unlike the location effect ($F = 0.01$, $P = 0.98$) and interaction ($F = 2.5$, $P = 0.12$). The nonparametric test also indicated a significant species effect ($H = 6.9$, $P < 0.05$), and insignificant location ($H = 0.2$, $P > 0.05$) and interaction ($H = 3.7$, $P > 0.05$) effects. Blue crabs had somewhat larger carapace widths than stone crabs (Table 4), but stone crabs with a carapace width of 9 cm are readily capable of feeding on oyster drills (Brown & Haight 1992). There were no significant differences in oyster drill shell lip thickness among locations (Table 4, $F = 0.5$, $P = 0.62$), although total shell length had a highly significant effect on shell lip thickness ($F = 18.3$, $P < 0.001$). The lack of a significant interaction ($F = 0.4$, $P = 0.7$) indicates no difference in slopes of lines relating lip thickness to shell length among locations, and the slightly greater lip thickness of oyster drills at the estuarine, subtidal oyster reef therefore appears primarily due to their greater average shell length (Table 4).

Growth Experiments

There were significant differences in growth among the three locations in both experiments, with higher growth rates at the subtidal, estuarine oyster reef. Although the repeated measures ANOVA of the winter 1998–1999 data detected no time (e.g., month) effect (Wilk's $\lambda = 0.53$, $F = 1.1$, $P = 0.45$), there was a significant interaction between time and location (Wilk's $\lambda = 0.062$, $F = 3.8$, $P < 0.05$), and a significant difference among locations ($F = 37.9$, $P < 0.001$). The covariate, initial size, also

TABLE 4.

Crab catch per unit effort for two species of crabs, crab carapace widths (in cm), and oyster drill shell lip thickness and total shell length (both in mm), at two locations along the Louisiana Gulf of Mexico coast.

Location	Trait	Blue Crab	Stone Crab	Drill Shell Thickness	Drill Shell Length
Fourchon Lab	CPUE	60.7 ± 46.6	2.3 ± 1.5	0.75 ± 0.04	55.2 ± 0.9
	Carapace width	12.8 ± 0.3 (182)	9.4 ± 0.2 (7)		
Caminada Pass	CPUE	45.7 ± 15.5	1.0 ± 0.2	0.57 ± 0.03	41.9 ± 0.5
	Carapace width	13.8 ± 0.1 (137)	8.5 ± 0.1 (3)		

All values are means and standard errors. Thirty oyster drills were measured at each site, and numbers in parentheses are the number of crabs measured. CPUE, crab catch per unit effort.

had a significant effect on growth ($F = 53.1$, $P < 0.001$). Portraying the results of this winter growth experiment as Ford-Walford plots (Fig. 2A) clearly illustrates the greater growth rates of smaller oyster drills at the subtidal oyster reef, as well as the significant effect of the covariate. The same basic pattern occurred in the summer 1999 experiment, although growth rates overall were somewhat lower (Fig. 2B). The repeated measures ANOVA again detected no time effect (Wilk's $\lambda = 0.79$, $F = 0.57$, $P = 0.65$), a significant interaction between time and location (Wilk's $\lambda = 0.066$, $F = 5.8$, $P < 0.01$), and a significant difference among locations ($F = 33.9$, $P < 0.001$). The covariate, initial size, again had a significant effect on growth as well ($F = 40.3$, $P < 0.001$).

Reproduction Experiment

There were clear differences between the two locations (with values again greater at the subtidal, estuarine oyster reef than at the exposed location) and oyster drill size classes in number of egg capsules deposited per cage (Fig. 4A). Both the location ($F = 29.7$, $P < 0.0001$), shell length ($F = 5.2$, $P < 0.01$), and location times shell length interaction terms ($F = 5.2$, $P = 0.01$) were significant in the ANOVA. Comparing Tukey's *a posteriori* tests among size classes within locations, there were no significant differences among size classes at Caminada Pass. However, at the subtidal, estuarine oyster reef, the largest snails (60 mm) overlapped with the next largest (50 mm) but not the two smaller size categories of snails, whereas the snails averaging 50 mm overlapped with the 40-mm snails but not the 30-mm size category, and the 40-mm- and 30-mm-sized snails overlapped as well. Comparing locations within size classes, oyster drills laid more egg capsules at the subtidal, estuarine oyster reef than at Caminada Pass in both of the larger size classes, but not in the smaller size classes.

Average number of embryos per capsule also varied significantly among locations (again greater at the subtidal, estuarine oyster reef, $F = 5.6$, $P = 0.03$) and size classes ($F = 31.1$, $P < 0.0001$), without any interaction (Fig. 4B). Oyster drills at the subtidal, estuarine oyster reef produced on the average 2,021 embryos per capsule versus 1,598 at Caminada Pass. *A posteriori* tests indicated the largest oyster drills produced significantly more embryos per capsule than the other three size classes, while the two intermediate classes overlapped, as did the 30- and 40-mm shell-length oyster drills. These values compare with average embryo numbers per capsule of about 3,200 for snails greater than 40-mm shell length held under laboratory conditions (Roller & Stickle 1988). Multiplying average numbers of egg cases times average embryo counts, and assuming five of the snails were females, per capita fecundity at Caminada Pass was approximately 35,000 versus 1,020,000 at the estuarine oyster reef.

Prey Addition Experiment

Addition of oyster prey to cages at the exposed site (= CP) resulted in both increased growth in oyster drill shell length ($F = 7.4$, $P = 0.02$) and higher production of egg capsules ($F = 11.5$, $P = 0.01$) in comparison to control cages. Growth in shell length

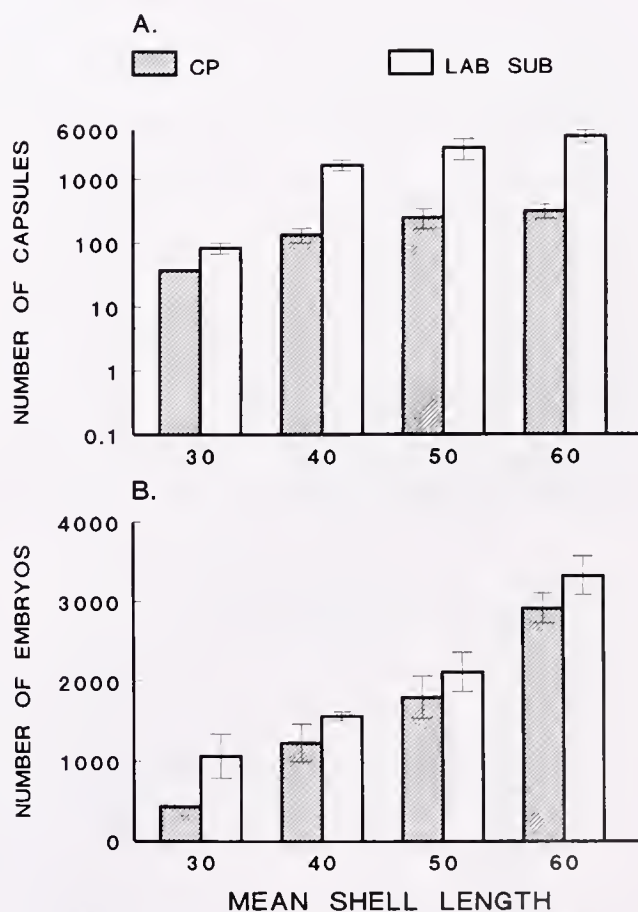


Figure 4. (A) Semi-logarithmic plot of size-specific differences in number of egg capsules deposited per cage ($\bar{x} \pm SE$, $n = 4$) at two locations along the Louisiana Gulf coast. CP = Caminada Pass, Laboratory Sub = subtidal oyster reef at Port Fourchon. The standard error is missing for the smallest snails at CP because only one cage had egg cases. (B) Size-specific differences in numbers of embryos per egg capsule per cage ($\bar{x} \pm SE$, $n = 4$) at two locations along the Louisiana Gulf coast. CP = Caminada Pass, Laboratory Sub = subtidal oyster reef at Port Fourchon.

was greater in experimental treatments by a factor of eight, and production of egg capsules increased by a factor of four (Fig. 5).

DISCUSSION

In general, intraspecific life history variation in intertidal gastropods is usually explained as the result of three factors acting singly or in combination: (1) differences in prey quantity or quality (Palmer 1983, Palmer 1984, Spight 1982), (2) differences in wave action (Brown & Quinn 1988, Denny et al. 1985, Richardson & Brown 1990), or (3) variation in predation risk (Palmer 1990, Richardson & Brown 1992). In *Stramonita haemastoma*, a combination of these factors probably explains variation among locations in life history patterns. The dominant prey at Caminada Pass, the barnacle *Balanus eburneus*, may not be as conducive to producing growth as oysters, the most common prey at the estuarine locations, explaining the increased growth and fecundity of the oyster drills in cages with added oysters at the exposed site. Although there is yearly variation in abundance, especially for barnacles, oysters were consistently more abundant at the estuarine sites over time in this study, and this same trend occurs at other locations along the Gulf of Mexico coast, and the lower Atlantic coast of the United States as well (Bahr & Lanier 1981, Brown & Swearingen 1998, Chatry et al. 1983, Roegner & Mann 1995, Wilber 1992). However, the increased tidal flows and wave action at the exposed site could also reduce growth (Richardson & Brown 1990), and the effects of food value and wave action are thus likely confounded. Increased wave action can also directly reduce average sizes in exposed populations by dislodging the largest individuals (Denny et al. 1985).

Although we worked with only one location within each exposure or tidal height category, we consider our results to have some generality because these trends to a large extent agree with other studies on this and other intertidal snail predators. Brown and Richardson (1987) recorded higher densities and smaller-sized individuals of *Stramonita haemastoma* at exposed locations in comparison to protected locations, and others have also found considerable variation in intertidal gastropod densities and size distributions among locations and seasons (Carroll & Highsmith 1996, Navarette 1996, Petraitis 1998). The greater growth at the subtidal,

estuarine oyster reef than at the exposed location is similar to earlier studies of *Nucella lapillus* (Etter 1988, Etter 1989, Etter 1996) and *Nucella emarginata* (Brown & Quinn 1988). However, Etter (1989) also found increased fecundity at exposed locations. Differences in growth rates in *N. lapillus* are the result of eco-phenotypic variation, not genetic differences among populations (Etter 1988, Etter 1996). *Stramonita haemastoma* has a relatively long pelagic larval stage, lasting 90 days, and there is considerable gene flow among populations in the northern Gulf of Mexico (Liu et al. 1991), arguing against high levels of genetic variation among these populations, which are only separated by tens of kilometers. The prey manipulation experiment also suggests that oyster drill growth and reproductive rates are plastic and will respond to increased food availability.

Oyster drills in the northern Gulf of Mexico reach 30 mm in shell length and usually reproduce in their first year (Butler 1985). However, the slow growth and small sizes attained by the oyster drills at Caminada Pass (average oyster drill size was only 35 mm) may mean they do not reproduce until their second spring, and senesce shortly afterwards. This could explain the peak recruitment at Caminada Pass in only 1 yr of the study, as a cohort matured, reproduced, and senesced. In contrast, at the subtidal protected oyster reef, rapid growth may have resulted in reproduction in the first year, and individuals probably survive and reproduce for several years (average size was around 50 mm, and iteroparity may also explain the bimodal size distribution at this site shown in Fig. 3). Such iteroparous reproduction has also been reported before in southern oyster drills (Butler 1985).

Our results indicate that wave exposure along the relatively protected Louisiana Gulf of Mexico coastline does not have as extreme an effect on life histories as it does in thaidid snails along other coastlines. Wave exposure may have a more indirect effect, by altering the abundances of prey organisms (oysters again recruit more readily at estuarine locations) or forcing oyster drills to consume smaller, suboptimal prey (Richardson & Brown 1990). Nor does predation risk appear as important as for thaidids along other coastlines, as we could detect no differences in the abundances of stone crabs among locations; in fact stone crabs were relatively rare in comparison to blue crabs, which are effective predators only on small, recently settled oyster drills (Butler 1985). If anything, predators like stone crabs or black drum are probably more abundant in the northern Gulf of Mexico at higher salinity coastal sites (Brown et al. 2003), and the increased predation risk seen at protected sites for thaidids on other coastlines either does not occur in this system or may even be reversed, with higher predation rates at coastal sites. The lack of any difference in shell lip thickness among locations also provides indirect support for the lack of any differential in predation risk among sites.

We conclude that prey value and availability appear most important in explaining intraspecific life history variation in southern oyster drills, as both growth and reproductive rates responded with four- to eight-fold increases when oyster prey were provided to snails held at exposed sites. This result is certainly consistent with other studies suggesting that prey value and availability may determine thaidid growth rates (Palmer 1983) and recruitment (Spight 1975, Spight 1982).

ACKNOWLEDGMENTS

Sean Keenan, Patrick Banks, and Susan Bolden helped with field work and Dr. James Geaghan consulted with us on statistical analyses.

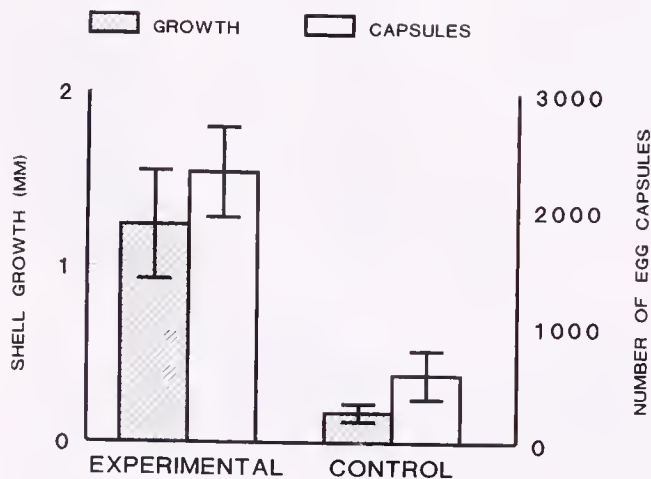


Figure 5. Mean growth in shell length (left y axis) and number of egg capsules produced (right y axis) per cage (\pm SE, control $n = 4$, experimental $n = 6$) in the experiment where oyster prey were added to cages at Caminada Pass.

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CAPTIVE BREEDING BEHAVIOR OF FOUR STROMBIDAE CONCH

AMBER L. SHAWL AND MEGAN DAVIS

Harbor Branch Oceanographic Institution 5600 US 1 North, Ft. Pierce, Florida 34946

ABSTRACT The feasibility of a captive breeding program was examined for the protected fisheries species *Strombus gigas* (queen conch) and three nonprotected Caribbean *Strombus* conch species: *S. raninus* (hawk-wing conch), *S. alatus* (Florida fighting conch), and *S. costatus* (milk conch). A total of 24 adult conch were collected from the Florida Keys: five *S. costatus* (three females, two males), seven *S. raninus* (five females, two males), eight *S. alatus* (four females, four males), and four *S. gigas* (one female, three males). The conch were placed in a 4.5-m dia circular tank that was divided into four equal quadrants (4.1 m²). The conch were fed a prepared gelatin diet made of Mazuri® Koi chow blended with *Ulva* sp. Egg masses collected from the breeding tank were measured for size, number of eggs, and diameter of egg capsule and strand. In the 40-week study, 426 egg masses were collected, and egg laying activity appeared to be related to water temperature. A total of 341 egg masses were collected from the five *S. raninus* females. The four *S. alatus* females laid 58 egg masses, the three *S. costatus* females laid a total of 23 egg masses, and the single *S. gigas* female laid four egg masses. The viability of egg masses from all four species was confirmed by successfully hatching and culturing the larvae to the juvenile stage. All species except *S. gigas* laid egg masses while copulating. Mate preferences and guarding behavior was observed. The captive breeding of these species holds promise for the establishment of a commercial captive breeding program, and may provide alternative conch species for the food and aquarium market.

KEY WORDS: conch, culture, larvae, reproduction, spawning, *Strombus*

INTRODUCTION

Six *Strombus* species¹ inhabit the shallow coastal waters of Florida and the Caribbean region: *Strombus gigas* Linnaeus (queen conch), *S. costatus* Gmelin (milk conch), *S. raninus* Gmelin (hawk-wing conch), *S. alatus* Gmelin (Florida fighting conch), *S. pugilis* Linnaeus (West Indian fighting conch), and *S. gallus* Linnaeus (rooster-tail conch; Abbott 1974). Of these species, *S. gigas* holds the highest commercial value as a subsistence and commercial fisheries product (Berg 1976, Brownell 1977, Appeldoorn 1994). *S. costatus* is also a highly valued fisheries species in Mexico (Aldana-Aranda et al. 1989).

Overfishing and the decline of the *S. gigas* fishery began in the 1970s and led to the enactment of several regulations and management strategies to protect conch populations (Appeldoorn 1994). A statewide moratorium on queen conch harvesting in Florida began in 1986 (Florida Administration Code, Chapter 68B-16.005), and in 1992, *S. gigas* was added to Appendix 2 of the Convention for the International Trade of Endangered Species (CITES) legal mandate. Countries that export queen conch must possess a CITES permit, which helps to ensure that harvest rates remain low enough to maintain a viable fisheries population. In addition to employing management strategies to protect wild populations, culturing queen conch for stock enhancement and commercial markets will also offset fisheries pressure (Creswell 1994, Davis and Shawl (2004a).

Florida and Caribbean *Strombus* species breed in shallow waters in seagrass beds or sandy habitats (Robertson 1959, Berg 1975, Brownell 1977, Bradshaw-Hawkins 1982, Davis et al. 1984). The reproductive season is typically during the summer months; however, some species have been observed to lay eggs year-round in certain locations (Robertson 1959, Brownell 1977, Bradshaw-Hawkins 1982). Depending on the species, the sand-covered, crescent-shaped egg mass contains between 92,000 and 400,000 eggs (Robertson 1959, Randall 1964, D'Asaro 1986, Davis et al. 1993).

Commercial and research facilities that culture juvenile queen

conch depend upon field collected egg masses (Davis 2000a). In some locations, it is difficult to find egg masses in the wild due to limited spawning aggregations. Establishing a captive breeding program for *S. gigas* and other *Strombus* species would alleviate the need to collect egg masses from the wild and may possibly extend the 6-month breeding season to year-round (Davis et al. 1984). Captive breeding and egg-laying has been observed for some *Strombus* conch species. The reproductive behavior and larval cycles of wild and captive *S. pugilis* have been described (Bradshaw-Hawkins 1982); captive laid egg masses from *S. raninus* broodstock were used to record shell morphology and larval development (Davis et al. 1993); and the reproductive behavior and anatomy of captive *S. raninus*, *S. gallus*, *S. costatus*, *S. alatus*, and *S. pugilis* were observed (Reed 1995a, 1995b). *S. gigas* have been observed to copulate in captivity (Davis, personal observation); however, there are no reports regarding egg mass laying in captivity.

Based on the available reproductive data, it is hypothesized that adult *S. gigas* and other *Strombus* species can reliably lay egg masses in captivity. Therefore, the goal of this study was to determine the feasibility of establishing a captive breeding program for *Strombus* species. To address this goal, the first objective of this study was to observe and describe the spawning activity of the protected fisheries species *S. gigas* and three nonprotected *Strombus* species: *S. raninus*, *S. alatus*, and *S. costatus* in a breeding tank. The second objective was to confirm the viability of the captive laid eggs by culturing the larvae to the juvenile stage. The results from this study will assist in developing a year-round culture program, alleviating the field collection of egg masses, and establishing alternative species for the *Strombus* markets.

MATERIALS AND METHODS

The captive breeding study was conducted at Harbor Branch Oceanographic Institution (Harbor Branch), Aquaculture Division in Ft. Pierce, Florida. Egg masses were collected for 40 weeks (June 15, 2000, to March 22, 2001), and breeding behavior observations were made daily during the first 7 weeks of the study (June 15 to August 2, 2000). Larvae hatched from the egg masses laid in captivity were cultured throughout the study period.

*Correspondence: ashawl@hboi.edu

TABLE 1.

Description of Upper Florida Keys collection sites for *Strombus* species.

Site No.	Location and Date	Depth (m)	Bottom Type	Water Temp (°C)	Salinity (ppt)	pH	Ammonia (mg/L)	Species	Sex	No.
1	Sea Oat Beach, Lower Maticumbe (June 15, 2000)	1.5	Fine sand, <i>Batophora</i> , <i>Laurencia</i> , <i>Halimeda</i> , <i>Thalassia</i> , <i>Syringodium</i>	29.7	36	8.2	0.06	<i>S. raninus</i>	M	1
2	Windley Cay, Coral Club (June 15, 2000)	0.16–1.5	Soft lime sand, fine diatom film, <i>Syringodium</i> , <i>Halimeda</i>	31.2	35	8.4	0.07	<i>S. raninus</i>	F	5
								<i>S. raninus</i>	M	1
								<i>S. alatus</i>	F	1
								<i>S. alatus</i>	M	3
3	Ligumuiti Channel, Indian Key ^a (June 1, 2000)	2–2.5	Sandy, rocky hard bottom	N/A	N/A	N/A	N/A	<i>S. costatus</i>	F	3
								<i>S. costatus</i>	M	2
4	Florida Fish and Wildlife Conservation Commission, Keys Marine Laboratory, Long Key, FL ^b (July 16, 2000)	0.75	Fiberglass holding tanks (0.75 m wide × 3.6 m length)	26–32	N/A	N/A	N/A	<i>S. gigas</i>	F	1
								<i>S. gigas</i>	M	3

^a *S. costatus* were collected and held in a recirculating system for 2 weeks prior to being transported to HBOI.^b Hatchery reared from *S. gigas* eggs collected in Florida waters.

The number of conch collected for this study was based on the availability of the conch at the collection sites. Seven adult *S. raninus* (two males and five females) and four *S. alatus* (three males and one female) were collected from three sites off Plantation Key in the Upper Florida Keys on June 15, 2000. On July 12, 2000, four additional *S. alatus* (one male and three females) were collected from the same location (Table 1). Five *S. costatus* (two males and three females) were collected on June 1, 2000, from the Ligumuiti Channel in Indian Key in the Upper Florida Keys and were placed in recirculating holding tanks in the Florida Keys for 2 weeks prior to the beginning of the study. Four hatchery-reared *S. gigas* (three males and one female) were obtained on July 12, 2000, from the Florida Fish and Wildlife Conservation Commission laboratory in Marathon, FL.

The sex of each conch was determined by positioning them on their side and observing a verge or an egg groove when it righted itself (Davis et al. 1984; Fig. 1). A small area on the dorsal side of the shell was then scrubbed clean with sandpaper and the conch were numbered with fluorescent paint (teal for males and orange for females), which was sealed with clear epoxy glue. The conch were transported in a cooler with seawater moistened towels during the four hour drive to Harbor Branch. Before being placed into the breeding tank at Harbor Branch, all of the conch were weighed to the nearest 0.1 g, and their shell lengths (SL) were measured with calipers (mm; Table 2).

The 11,500-L breeding tank (4.5 m dia × 0.7 m depth) was on a recirculating culture system (Fig. 2). The initial and replenishment water (30 L/hr or 6.5%/d) for the system was pretreated water drawn from a shallow salt water well. For the study, the breeding tank was divided into four equal areas (4.1 m²) using 30-cm high polypropylene mesh (2.5-cm openings). The tank was equipped with a raised substrate comprised of approximately a 10-cm depth of sand supported 15 cm above the bottom of the tank. The crushed coral aragonite sand (1–3-mm dia) provided substrate for the broodstock to lay egg masses and served as the biofilter for the system. The water depth above the substrate was 45 cm. The

recirculating water entered the tank both through angled spray bars above the water and substrate and underneath the substrate. The water filtered through the sand, drained through a center bottom drain, then flowed through a sand filter (Baker Hydro 363 kg) prior to returning to the breeding tank. The sand filter was backwashed every 2 weeks.

The conch were stocked at varying densities and sex ratios based on the availability of the adults found at the collection sites. *S. raninus* conch were held at 1.7 conch/10 m² at a 3:1 female: male ratio, *S. alatus* were stocked at 2.0 conch/10 m² at a 1:1 sex ratio, *S. costatus* were at 1.2 conch/10 m² at a 1:1 ratio, and *S. gigas* were held at 0.98 conch/10 m² at a 1:3 female: male sex ratio.

Temperature, salinity, and pH were recorded daily and ammonia was measured weekly using a HACH® Ammonia test kit

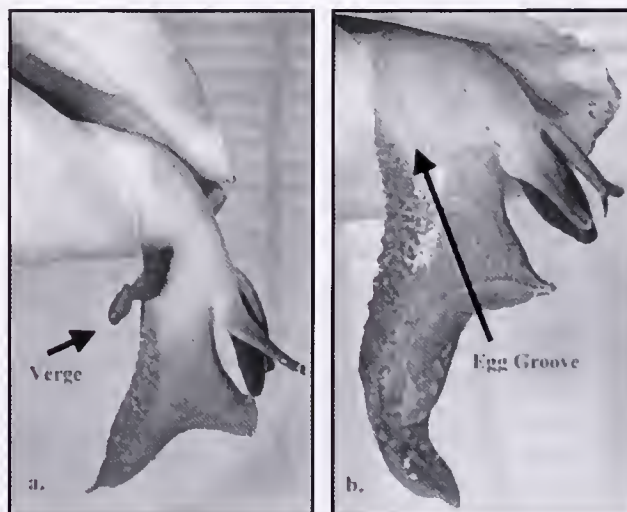


Figure 1. External sex organs for the conch family Strombidae. Shown here is the (a) male verge and (b) female egg groove for the milk conch, *Strombus costatus*.

TABLE 2.
Shell length (SL) and weight of adult *Strombus* conch collected from the Florida Keys.

Species	Sex	No. of Conch	SL Range (mm)	Avg. SL (mm)	Weight Range (g)	Avg. Weight (g)
<i>S. raninus</i>	M	2	72.8–74.9	73.9	63–72	67.5
	F	5	77.0–86.4	82.8	67–102	85.8
<i>S. alatus</i>	M	4	76.0–92.7	86.7	51.1–95.4	70.2
	F	4	80.2–94.7	89	70.7–109.8	86.8
<i>S. costatus</i>	M	2	170–173	172	754–776	765
	F	3	165–195	180	965–1100	1030
<i>S. gigas</i>	M	3	180–190	183.3	1094–1560	1267
	F	1	190	190	1151	1151

(Model NI-SA). The breeding tank was located within a shaded greenhouse, and natural light was supplemented with artificial lighting (250 watts) for a 12-h cycle (8 am to 8 pm). In an attempt to extend the breeding season, the tank water was heated using a 4000-watt (Titanium Single Phase "L"-Shaped Bottom Heater) heater during the winter months (November 2000 to March 2001).

The conch were fed a diet of Mazuri® Koi pellets, which were blended with dry *Ulva* sp. (sea lettuce), gelatin, and seawater to create a gelatin based benthic diet (R. LeRoy Creswell, personal communication 2000). The recipe was composed of 36% Mazuri Koi pellets, 16% *Ulva* sp., 42% seawater, and 5% gelatin. The gelatin allowed the food to remain stable for approximately 48 h. The conch were fed to satiation once per day. On average, a total of 745–925 g of gel food was fed once daily, with approximately 145 g per day fed to all the *S. raninus* conch, 150 g fed to the *S. alatus* conch, 300 g fed to *S. costatus*, and 240 g fed to the *S. gigas* conch.

Egg mass production was recorded daily. During the first seven weeks, copulatory behavior observations were made approximately five times per 24 h. Each observation spanned 15 min to one hour and included periodic night observations. The number of copulations, mate preferences, and any territorial male behavior were recorded. When an egg mass was found, it was removed with a net or by hand and placed onto a wet table for length, width and height measurements. Several egg masses were uncoiled to estimate the total length of the egg strand ($n = 13$ for *S. raninus*, $n = 10$ for *S. alatus*, and $n = 10$ for *S. costatus*). None of the *S. gigas* egg masses were uncoiled because of the limited number of masses

and the need for eggs for larval production. Measurements of egg strand diameter ($n = 5$ per egg mass), egg capsule diameter ($n = 5$ per egg mass), and the number of eggs per millimeter ($n = 3$ per egg mass) were recorded using a compound microscope (40X) equipped with an eyepiece micrometer. A total of 71 egg masses were used for this data collection; 40 for *S. raninus*, 20 for *S. alatus*, nine for *S. costatus*, and two for *S. gigas*.

Larvae of *S. raninus* (two egg masses), *S. alatus* (six egg masses), and *S. costatus* (one egg mass) were hatched from captive laid egg masses. The larvae were cultured at 20–50 veligers/L in two to three 5-L containers using established techniques (Davis 2000b). The culture water was 5- μ m filtered, UV-treated, and changed daily. The cultures were not aerated, and temperature was controlled by placing the containers in a water bath. Depending on age, the veligers were fed 3,000–20,000 cells/mL of culture water of Tahitian Isochrysis daily.

One *S. gigas* egg mass was cultured in a 420-L conical tank (Davis and Shawl, in press). The culture water was 5- μ m filtered and UV treated. An airlift provided circulation in the tank, and the water was changed every other day. The larvae were fed 5,000–25,000 cells/mL of culture water of Isochrysis, and starting on day 15, *Chaetoceros gracilis* or *Chaetoceros muelleri* was also added to the tank at a concentration of 3,000 cells/mL of culture water.

Metamorphosis was induced for all four species using diluted 3% hydrogen peroxide (Boettcher et al. 1997) or *Laurencia poitei* seaweed extract (Davis et al. 1987). A total of 529 larvae were tested; 305 larvae were induced with hydrogen peroxide, and 224 were induced with *Laurencia* extract. The SL of the newly hatched veliger (μ m), the number of days until metamorphosis, and the veliger SL at metamorphosis (μ m) was recorded along with the metamorphic success using the two inducers.

RESULTS

From June 15, 2000, to March 22, 2001 (40 weeks), a total of 426 egg masses were collected from the breeding tank. The five *S. raninus* females had the highest productivity with 341 masses, the four *S. alatus* females laid 58 masses, 23 egg masses were collected from the three *S. costatus*, and the single *S. gigas* female laid 4 egg masses. The weekly fecundity of each individual female conch was averaged. The number of egg masses per female per week per species was plotted and compared with average weekly temperature data (Fig. 3). In general, egg mass production was continuous during the warmer months, (26–29°C) June to September (Fig. 3). In the winter months, October to January, egg mass production slowed down or stopped when the temperature decreased (22–25°C). Egg mass laying began again in February

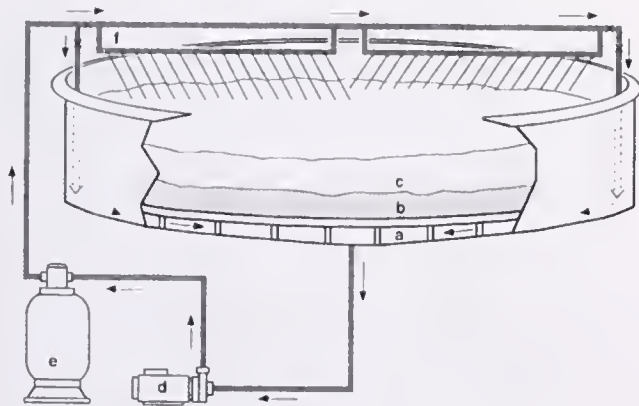


Figure 2. The Strombidae breeding tank at Harbor Branch: a) undergravel support, b) sand substrate, c) water column, d) pump, e) sand filter, and f) spray bars (drawing by Jackie Aronsan).

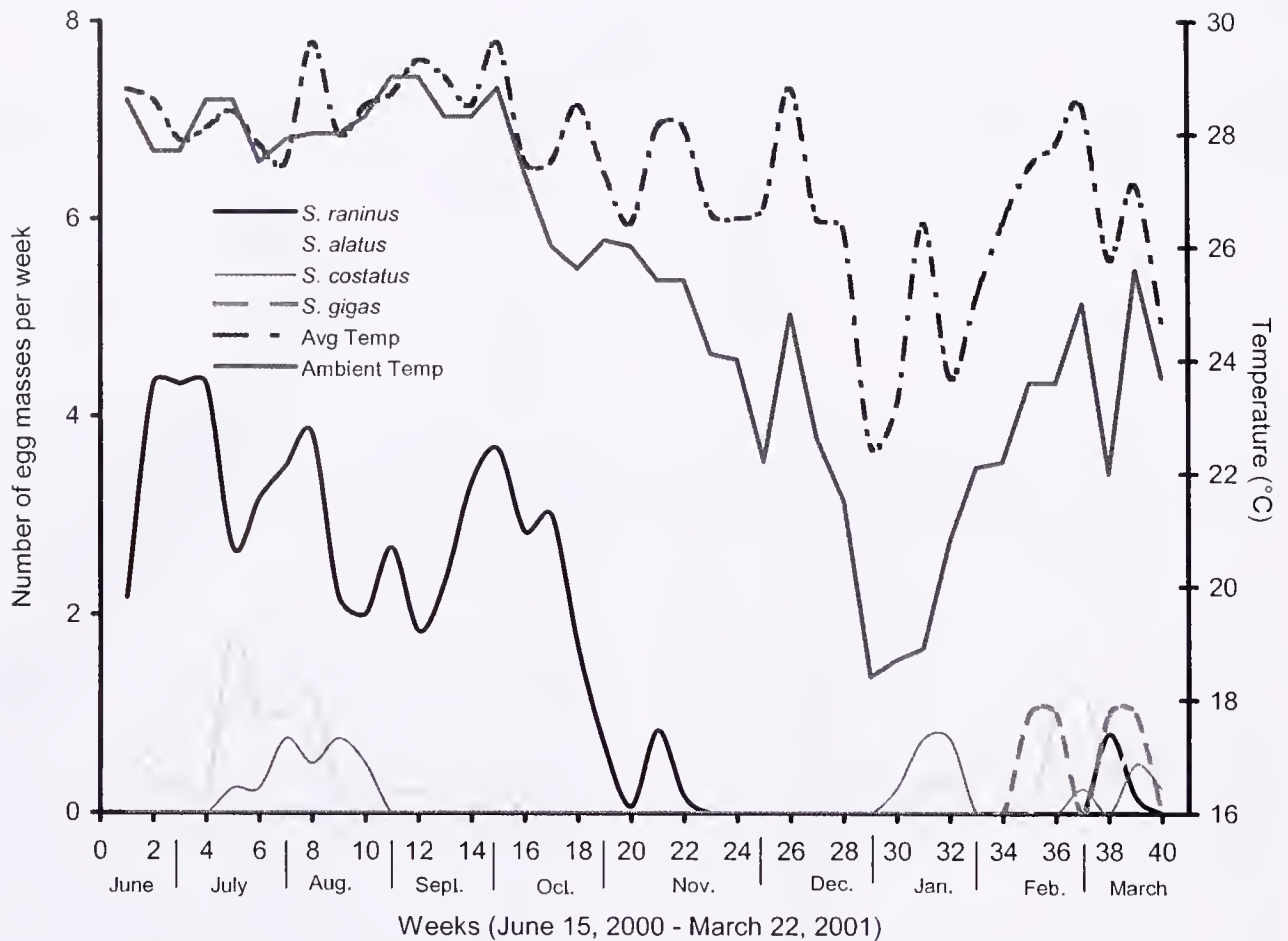


Figure 3. The weekly average number of egg masses collected from each female conch compared with weekly average tank and ambient temperatures for the duration of the captive breeding study at Harbor Branch (June 15, 2000, to March 22, 2001).

2001 when the temperature increased (25–29°C). By heating the breeding tank water from November 2000 to March 2001, the temperature remained 1–4°C above ambient water temperature (Fig. 3). This appeared to stimulate egg laying in the winter months (February to March 2001), as no other parameters were changed. Salinity, pH, ammonia, and nitrite remained relatively constant throughout the study (Table 3).

TABLE 3.

Water quality for the broodstock tank and the replenishment water

	Tank Water (June 2000 to March 2001)	Replenishment Water (June 2000)
Temp (°C)	27.4 ± 2.11 (277)	28.3 (1)
Salinity (ppt)	33.5 ± 1.25 (555)	33 (1)
pH	8.1 ± 0.28 (242)	7.7 (1)
Ammonia (mg/L)	0.1 ± 0.02 (39)	0.08 (1)
Nitrite	0.009 ± 0.006 (6)	—
Ca ⁺ ion Concentration (mg/L as CaCO ₃)	429 ± 12.7 (2)	403 (1)
Dissolved oxygen	6.2 (1)	4.09 (1)
ORP	203.1 (1)	195 (1)

Results are expressed as average ± standard deviation (n = samples). oxygen reduction potential

The weekly number of egg masses laid per species per week was standardized for each 10-week period of the 40-week study (Table 4). *S. raninus* females were the only species to lay egg masses throughout the four 10-week sample periods, with a maximum of seven egg masses collected from one female per week. The maximum number of egg masses laid from one female per week from *S. alatus* and *S. costatus* was five and three, respectively. The single *S. gigas* female did not lay an egg mass until

TABLE 4.

Weekly egg mass production during the 40-week study period, standardized as egg masses per female per week.

Weeks	<i>S. raninus</i>	<i>S. alatus</i>	<i>S. costatus</i>	<i>S. gigas</i>
1–10	3.25 ± 0.95 (7)	0.66 ± 0.57 (4)	0.30 ± 0.31 (3)	0
11–20	2.21 ± 1.16 (6)	0.06 ± 0.10 (1)	0	0
21–30	0.10 ± 1.15 (2)	0	0.03 ± 0.08 (1)	0
31–40	0.08 ± 0.21 (3)	0.44 ± 0.43 (5)	0.25 ± 0.31 (1)	0.67 ± 0.52 (1)

Results are expressed as average ± standard deviation. The number in parentheses represents the maximum number of egg masses collected from one female during a 1-week period.

TABLE 5.

Summary of egg mass data from the study (bold numbers) compared to published data.

Variable	<i>S. raninus</i>	<i>S. alatus</i>	<i>S. costatus</i>	<i>S. gigas</i>
Length of egg mass (cm)	8.4 ± 2.4 (315) 4–15.5 (315) 4–7 (4) ^c	6.5 ± 1.3 (30) 4.5–9 (30) —	15 ± 7.1 (10) 9–21.5 (10) 6–10 (2) ^c	9 (1) — 8–15 (9) ^c
Width of egg mass (cm)	21.1 ± 0.5 (315) 1.1–3 (315)	2.9 ± 0.6 (30) 1.5–4 (30)	4 ± 0.6 (10) 2.5–4 (10)	— —
Length of uncoiled strand (m)	7 ± 2 (13) 4.1–10 (13) 20 (1) ^a	10.8 ± 1.4 (10) 7.1–11.9 (10) 10.7 (10) ^b	19 ± 9.7 (9) 7.5–35.8 (9) 14.1 ± 1.3 (2) ^a	— — 22.6 (1) ^a 24–37 (10) ^d
No. eggs/mass (estimated)	91,000–250,000 (13) 206,000–245,000 (2) ^c 400,000–460,000 (1) ^a	76,000–182,000 (10) 92,000 (2) ^b	87,000–440,000 (9) 185,000–210,000 (2) ^a	— 313,000–485,000 (10) ^d 385,000–430,000 (1) ^a
Diameter of egg strand (μm)	351 ± 24 (40) 321 ± 20 (10) ^c	509 ± 41 (20) 600 (10) ^b	825 ± 56 (9) 761 ± 18 (10) ^c	798 ± 18 (2) 785 ± 44 (10) ^c
No. of egg capsules per mm	24.9 ± 3.1 (40) 20–23 (1) ^a 21–25 (15) ^c	12.3 ± 1.2 (20) 8.6 (10) ^b	12 ± 1.4 (9) 14 (2) ^a 12–14 (10) ^c	13.4 ± 0.9 (2) 17–19 (1) ^a 14–16 (10) ^c 12–15 (10) ^d
Egg capsule diameter (μm)	123 ± 10 (40) 140 ± 4 (30) ^a	181 ± 11 (20) 170 (10) ^b	250 ± 9 (9) 262 ± 6 (20) ^c	233 ± 11 (2) 225 ± 17 (20) ^c

Results are expressed as average ± standard deviation (n = samples) and ranges.

^a Robertson (1959).^b D'Asaro (1986).^c Davis et al. (1993).^d Randall (1964).

February 2001. One egg mass was collected each week during weeks 35, 36, 38, and 39.

Egg mass characteristics of the eggs laid by the captive adults were similar to those of egg masses laid in the wild (Table 5). *S. raninus* egg masses were long and narrow and consisted of a very thin and fragile egg strand ($351 \pm 24 \mu\text{m}$ dia). The *S. alatus* egg masses were shorter and wider than *S. raninus* and the egg strand was slightly larger in diameter ($509 \pm 41 \mu\text{m}$). *S. costatus* and *S. gigas* produced the largest egg masses with the thickest egg strands ($825 \pm 56 \mu\text{m}$ and $798 \pm 18 \mu\text{m}$, respectively). The *S. raninus* and *S. alatus* egg masses contained an estimated 76,000–250,000 eggs per mass, whereas the *S. costatus* egg masses held up to an estimated 440,000 eggs. The egg capsule diameter and number of eggs per mm appeared to be related to the size of the egg strand diameter. *S. raninus* egg capsules were small, therefore, each mm of egg strand contained approximately twice as many eggs as the other three species (Table 5). Females from all three species were observed to copulate consecutively with more than one male while laying the same egg mass.

From June 16 to August 2, 2000, the first 7 weeks of the study, copulatory frequency and reproductive behavior was observed for *S. raninus*, *S. alatus*, and *S. costatus*. The number of observed copulations was compared with the number of egg masses collected during that time period (Fig. 4). For *S. raninus*, the number of egg masses collected from the female conch was higher than the number of copulations observed. However, for *S. alatus* and *S. costatus* there were less egg masses collected compared with the number of copulations observed. All three species were observed copulating while laying an egg mass (Fig. 5). *S. alatus* were observed to display this behavior more frequently (52%), compared with *S. raninus* (31%) and *S. costatus* (19%; Fig. 5).

Several conch showed preferences for particular mates (Fig. 6). Using the males as examples, *S. raninus* male 1 showed no preference; he mated approximately an equal amount of the time with each of the five females. In contrast, *S. raninus* male 2 demonstrated a mating preference towards females 2 and 3. *S. alatus* male 1 showed a preference towards females 1 and 3, and male 2 showed preference towards females 3 and 4. *S. alatus* male 3 showed no preference and mated with all four females, whereas male 4 only mated with female 3. *S. costatus* male 1 copulated with all three females with approximately the same frequency; whereas, male 2 showed a preference towards female 2.

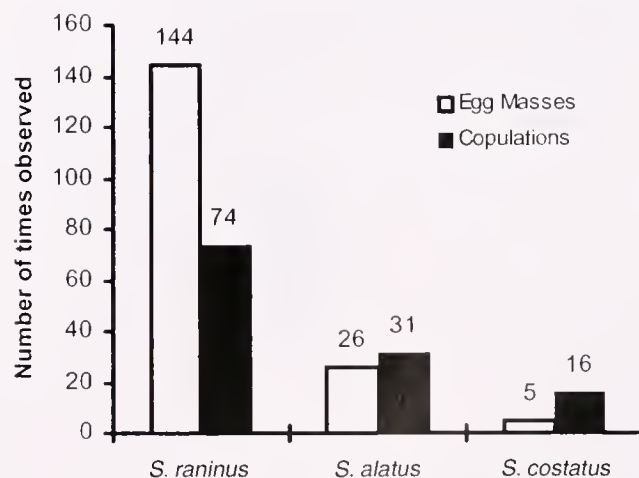


Figure 4. The total number of conch egg masses collected compared with the number of copulations that were observed from June 15 to August 2, 2000 (7 weeks).

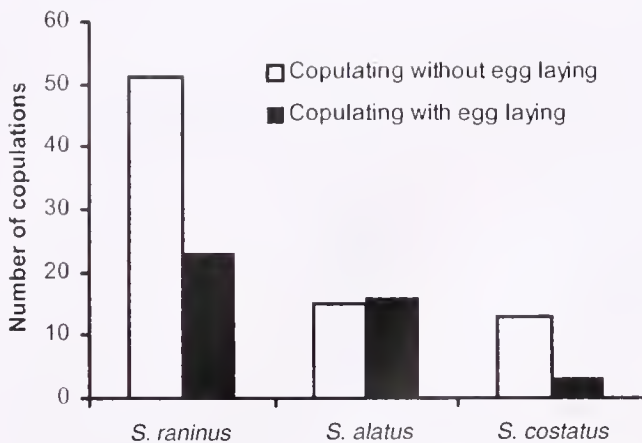


Figure 5. The number of times a female was observed simultaneously laying an egg mass while copulating versus the number of times a female was copulating and not laying an egg mass. Observations were recorded from June 15 to August 2, 2000 (7 weeks).

Conch were observed to copulate from 5 minutes to 2 h. The males would approach the females with their proboscis to initiate an interaction. Several times the females would leap away from the male, rejecting copulation. During other observations the males would follow the females around the perimeter of the enclosure before copulation was successful. There appeared to be no trend in mating pairs based on the size of the copulating female or male.

The *S. alatus* male conch in the breeding tank demonstrated protective courting behaviors. On several occasions, two males would prop themselves halfway on the lip of an egg-laying female. Only one of the males mated with the female while the other male appeared to be "guarding" the female. Behavior similar to this was observed in *S. pugilus* (Bradshaw-Hawkins 1982). The guarding male almost always had their siphonal canal positioned 90° to and on top of the female's dorsal side. During one observation, an egg-laying female had three aggressive males around her. One of the males guarding her used his proboscis in an attempt to dislodge the male that was mating with her. When the guarding male was unable to remove the mating male, he proceeded to use his proboscis to "spar" with that male. After a few minutes, the males stopped and the guarding male attempted to move back into his position. However, as the two males were sparring over top of the female, another male moved in. The third male was immediately chased away by the guard male. The guarding male then attempted to dislodge the mating partner once again, but was unsuccessful and copulation continued. The *S. alatus* broodstock were initially stocked at a 1:1 sex ratio. A few weeks into the study, the ratio in the breeding tank was increased to 1:2.5 female: male. Once male guarding and sparring were observed, some of the males were removed to decrease the sex ratio back to 1:1 to reduce stress on the egg-laying females.

All four captive species laid viable eggs in captivity. *S. alatus*, *S. costatus*, and *S. gigas* larvae were competent for metamorphosis in 18–24 days, and *S. raninus* were competent in 48 days (Table 6). When *S. alatus* larvae were exposed to the hydrogen peroxide inducer, $69.3\% \pm 17.2$ of the larvae metamorphosed, which was similar to larvae exposed to the Laurencia inducer ($62.5\% \pm 10.8$). The *S. costatus* larvae showed a low metamorphic success rate with hydrogen peroxide ($31\% \pm 14.1$) and an even lower rate with Laurencia ($0.7\% \pm 1$). However, the larvae that did not undergo metamorphosis with the Laurencia extract were reinduced three days later with hydrogen peroxide and 78% of these larvae com-

pleted metamorphosis. *S. gigas* larvae were only induced with hydrogen peroxide and demonstrated a metamorphic success rate of 69%. *S. raninus* larvae did not have a very high metamorphic success rate with either of the inducers ($< 7\%$).

Veliger shell length (μm) at hatch and at metamorphosis is shown in comparison with other laboratory cultured *Strombus* veligers (Table 6). *S. raninus* veligers hatched at the smallest size ($205 \pm 10.5 \mu\text{m}$) and *S. costatus* veligers had the largest SL at hatch ($370 \pm 10.5 \mu\text{m}$). The *S. gigas* larvae hatched at a much smaller SL than previously observed (Davis et al. 1993). The two smaller *Strombus* species, *S. raninus* and *S. alatus* veligers, had the largest SL at metamorphosis ($1438 \pm 72.2 \mu\text{m}$ and $1539 \pm 186 \mu\text{m}$, respectively), although *S. alatus* larvae were competent for metamorphosis in nearly half as many days. In contrast, previous studies have reported a much larger *S. costatus* veliger at the time of metamorphosis (Brownell 1977; Table 6).

DISCUSSION

Culturing conch in captivity can reduce the harvest pressure that is evident in the fishery (Berg 1976, Brownell 1977, Creswell 1994). All four conch species used in this study spawned in captivity in a recirculating tank system. This significant breakthrough means that the culturing cycle for *S. gigas*, *S. raninus*, *S. alatus*, and *S. costatus* can be closed. Potential conch farms may no longer need to rely upon wild reproductively active adult conch populations for egg masses. Raising conch in a recirculating system minimizes effluent discharge and also suggests that future conch aquaculture sites may not need to be located near seawater sources.

The success of spawning conch in captivity was caused by several factors. The conch were provided with a stable breeding site that was devoid of predators, had optimal water quality conditions, a consistent supply of food, and a sand substrate. Temperature also appeared to regulate spawning activity. In wild adult populations, increasing temperatures throughout the summer months along with a 12-h photoperiod characterizes the reproductive season for *S. gigas* (Stoner et al. 1992). When temperature was increased artificially in early February 2001, all four species began to lay eggs again. This is typically two months sooner than spawning occurs in the wild (Davis et al. 1984). In this study, the male and female conch had been artificially aggregated, which increased the potential for mating. Wild *Strombus* adult populations are usually found at a sex ratio of 1:1. The five *S. raninus* adults produced the majority of the egg masses with only two males. This may suggest that a breeding tank can be stocked with a higher female sex ratio while still maintaining an appropriate stocking density within the breeding tank.

The *Strombus* larvae hatched from the egg masses spawned in captivity were viable. Larvae from all four species became metamorphically competent and the hydrogen peroxide inducer yielded the highest percent metamorphosis. It appears that the *S. gigas* larvae hatched at a smaller size than previously reported, which could be a result of eggs laid in an artificial habitat. *S. raninus* females were the most prolific egg layers of the four species; however, the larval cycle was twice as long as the other species. *S. alatus* larvae had the shortest larval cycle and the highest tolerance for temperature and salinity changes.

This study has demonstrated the feasibility of establishing a captive breeding program for *Strombus* species. Due to the success of this study, an expanded captive breeding area and conch hatchery was developed and is in operation at Harbor Branch to culture *S. alatus* at a commercial scale (Davis and Shawl, 2004b), and *S. gigas* for conservation research.

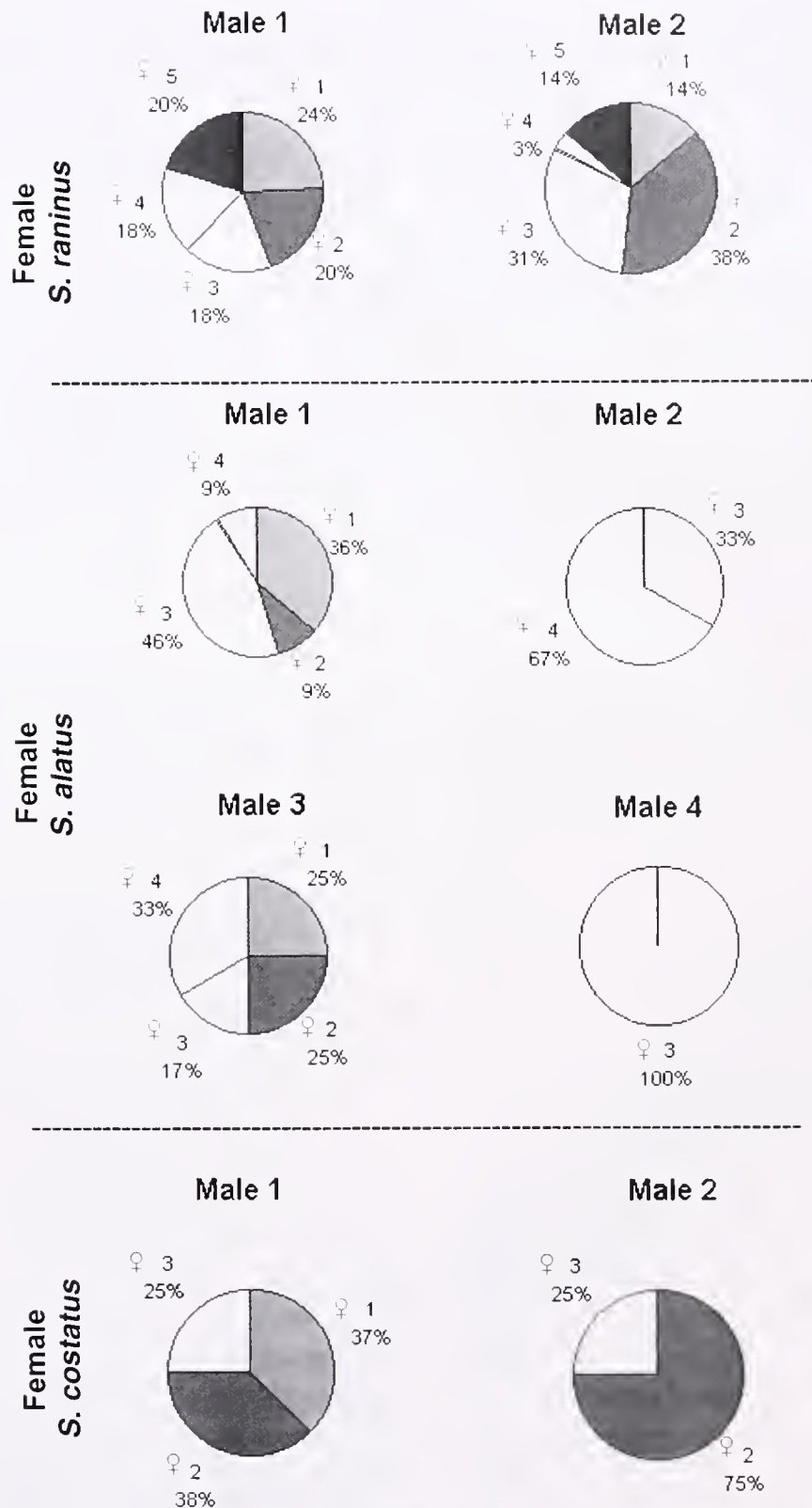


Figure 6. Male preferences towards female breeding partners. Observations were recorded from June 15 to August 2, 2000 (7 weeks).

To ensure reliable egg mass production in captivity, research must be conducted on photoperiod, temperature, density, nutrition, and sex ratio. Determining these parameters and cultivating

alternative *Strombus* species will assist in alleviating the need to collect egg masses from the wild, provide year-round egg masses, and will ultimately relieve pressures on the *S. gigas* fishery.

TABLE 6.

Summary of veliger and metamorphosis data from the study (bold numbers) compared to published data.

Variable	<i>S. raninus</i>	<i>S. alatus</i>	<i>S. costatus</i>	<i>S. gigas</i>
Newly hatched veliger SL (μm)	205 \pm 10.5 (10) 197 \pm 8 (20) ^a	298 \pm 14.2 (10) —	370 \pm 10.5 (10) 388 \pm 14 (20) ^c	221 \pm 28.4 (20) 354 \pm 15 (20) ^c
No. of days to metamorphosis	48 (1) 40 ^a	24 (1) —	18 (1) 15–18 ^b 32 ^c	18 (1) 18–21 ^b 21 ^c
Veliger SL at metamorphosis (μm)	1438 \pm 72.2 (4) 1450 \pm 53 (10) ^c	1539 \pm 186 (55) —	1306 \pm 23.9 (23) 4000 (31) ^b 1277 \pm 101 (10) ^c	1281 \pm 63 (10) 1240 (69) ^a 2200 (37) ^b 1170 \pm 58 (10) ^c
Metamorphic success (%)				
Hydrogen peroxide	5 \pm 2.3 (2)	69.3 \pm 17.2 (3)	31 \pm 14.1 (2)	68 (1)
<i>Laurencia</i>	6.6 (1)	62.5 \pm 10.8 (4)	0.7 \pm 1 (2)	—
Reinduction with H ₂ O ₂	—	—	78 (1)	—
Number of larvae per sample size	30	12	70, 76, and 111	69

The results are expressed as average \pm standard deviation (n = samples).

^a Berg (1976).

^b Brownell (1977).

^c Davis et al. (1993).

ACKNOWLEDGMENTS

The authors would like to thank the following individuals for their contributions to this study: Richard Greenfield, Jr., from CaribSea, Inc.; Buddy and Joe Pinder from the Keys Marine Conservancy; Robert Glazer from the Florida Fish and Wildlife Conservation Com-

mission; and Jerry Corsaut, Tom Smoyer, Brian Cousin, Jackie Arosan, Tina Powell, and Riccardo Russo from Harbor Branch Oceanographic Institution, Inc. We would also like to thank John Scarpa, Ken Riley, Ashley Spring, and an anonymous reviewer for their editorial comments. This research was partially supported by The Link Foundation. This is a Harbor Branch contribution 1546.

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GENETIC HETEROGENEITY ANALYSIS AND RAPD MARKER DETECTION AMONG FOUR FORMS OF *ATRINA PECTINATA* LINNAEUS

XIANGYONG YU,^{1,2} YONG MAO,² MEIFANG WANG,² LI ZHOU¹ AND JIANFANG GUI^{1,*}

¹State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China and ²Fisheries College, Zhanjiang Ocean University, Zhanjiang 524025, China

ABSTRACT Pen shell (*Atrina pectinata* Linnaeus) can be distinguished into four forms based on the morphologic characteristics. Genetic similarity and heterogeneity were analyzed among the four forms by random amplified polymorphic DNA (RAPD) technique using 24 10-nucleotide-long primers. Of these primers, 22 primers produced well-identifiable RAPD band patterns. Significant differences in RAPD band patterns were revealed among the four forms. A total of 198 polymorphic fragments were scored from 22 primers, and they are specific for one form, shared by two or three forms. Several primers, such as S451, S453, S463, S464, S470, S473, and S474, produced abundant band patterns and provided sufficient information for reliable discrimination of the four forms. The average genetic distances and phylogenetic relationships were calculated and analyzed according to the distinguishable fragments. The data indicate that pen shells of form G and form Y are similar not only among individuals within the same form, but also between individuals from the two forms, and that shells of form T and form S are highly divergent. The constructed phylogenetic tree matches the average genetic distances. Three clusters were clearly distinguishable, in which two were corresponding to form S and form T respectively and one included forms G and Y. This study will be benefit to further studies on the taxonomy and selective breeding of Pinnid species. It is suggested that the four forms of pen shell should be categorized to at least two species taxonomically.

KEY WORDS: RAPD marker, genetic polymorphism, taxonomy, *Atrina pectinata*

INTRODUCTION

Marine shells of the family Pinnidae are a popular food source and of high commercial value in a number of Asia-Pacific countries. In China, there are a number of Pinnid species that are presently categorized into three subgenera. The pen shell, *Atrina pectinata* Linnaeus, is the most commercially prominence of these species and is the only Pinnid species found in both northern and southern China waters (Wang 1997). In the South China Sea, including Baibu Gulf, *A. pectinata* shows considerable morphologic variation, which has produced considerable taxonomic confusion for more than 40 y (Wang 1964, 1997, Liang et al. 1986, Bernard et al. 1993). In an attempt to characterize its diversity and analyze its population structure, two methods, morphologic comparison and isoenzyme electrophoresis, have been used. These studies have revealed significant variations in not only exterior shell character and size of posterior adductor muscle (Yu et al. 2000), but also in the isozyme electrophoretograms (Wang et al. 2000).

According to their morphologic characteristics, especially the traits of exterior shell, *Atrina pectinata* in the South China Sea, can be divided into four forms: “green pen shell” (form G), “yellow pen shell” (form Y), “thorny pen shell” (form T), and “scabrous pen shell” (form S), respectively (Fig. 1). In areas such as the Leizhou Peninsula, collections of shells from four sites have shown that the four forms are sympatric. In collections from Donghai Island and Wailuo, all four forms occur in similar numbers, whereas at Shatian and Qintou, forms G and Y are most common, with only a few individuals of form T or S (Fig. 2; Yu’s personal observation). Electrophoretic comparisons of isozymes from different tissues have indicated that there are differences in the isozyme phenotypes among the four forms, which are consistent with the morphologic forms. This electrophoretic analysis has

shown that forms G and Y are analogous, while form S differs markedly (Yu et al., 2000; Wang et al., 2000).

The random amplified polymorphic DNA (RAPD) technique is a rapid and sensitive polymerase chain reaction (PCR)-based method (Williams et al., 1990) that has been widely used in polymorphism analysis for genetic structures. The RAPD technique had been shown to be a powerful tool for discrimination of different aquatic species or subspecies and for genetic analysis of phylogenetic relationships among strains or populations (Zhou & Gui 2000a, 2000b, 2002, Gui, 2003). In marine bivalves, RAPD techniques have been used to analyze genetic variation in oysters (Liu et al. 1998, Liu & Dai 1998), mussels (Kimura et al. 1997) and scallops (Patwary 1994), and proved an appropriate tool at molecular level for identifying divergence between populations or sibling species.

In this study, the RAPD technique was used for analyzing genetic diversity in the four forms of *Atrina pectinata*. We sought to reveal whether the four forms of pen shell distinguished morphologically could also be categorized by RAPD analysis. One of aims was to select suitable strains for farming because several traits differ among the four forms, such as the comparative size of adductor muscle, which is related to consumer popularity and market price in China. Another main aim is to provide more convincing evidence for the taxonomic categorization of the different forms of pen shell.

METHODS

Source of Samples

A total of 85 individuals of the four forms that caught from coast waters of Zhanjiang and nearby areas, in southern China (Fig. 2), were used for morphologic comparison. Because preliminary experiments demonstrated that most individuals in same forms produced basically identical RAPD band patterns, only eight adult individuals selected from each form were used to analyze genetic similarity and heterogeneity and to seek the specific markers for discriminating different forms.

*Corresponding author. E-mail: jfgui@ihb.ac.cn

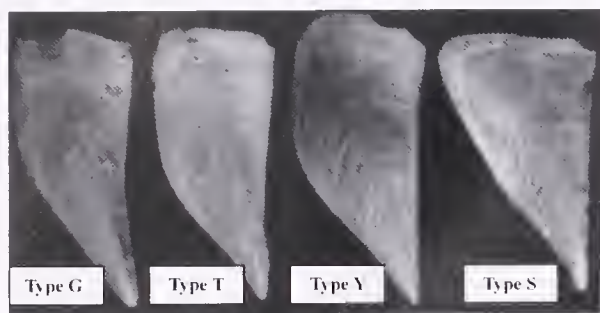


Figure 1. Four forms of *Atrina pectinata* distributed in Southern China Sea. Type G: green pen shell; type T: thorny pen shell; type Y: yellow pen shell; type S: scabrous pen shell.

Morphologic Comparison

Morphologic comparison was performed by observing the shell exterior and partially anatomic characters, such as the shell surface status, the radial sculpture situation, scale, the color of pallial organ and kidney, the ratio of shell height/shell breadth, and so on.

DNA Extraction

Total genomic DNA was isolated from ovarian tissue using standard phenol-chloroform extraction procedures (Sambrook et al., 1989). Briefly, about 1.5 g of tissue from each sample was homogenized into 15 mL of homogenized buffer (10 mM Tris-HCl, pH 8, 75 mM NaCl, 5 mM EDTA, 0.5% SDS) at 4°C. RNase was added and the suspension was incubated for 1 h at 37°C. 100 $\mu\text{g}\cdot\text{mL}^{-1}$ of proteinase K was then added and the suspension was again incubated for 2 h at 55°C. Proteins and other impurities were removed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1). The DNA was precipitated with 2 volumes of cold ethanol, 1/10 volume 3 M NaAc and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). After quality and concentration, samples were determined by agarose electrophoresis as described (Zhou et al. 1998), the DNA samples were stored at -80°C.

RAPD-PCR

Because RAPD analysis is particularly sensitive to the concentration of DNA, magnesium, primer, and Taq DNA polymerase, the optimal reaction conditions must be determined before study (Black 1993, Williams et al. 1993). In our system for RAPD amplification of the four forms of pen shells, we adopted the condi-

tions at the raised annealing temperature of 39°C, which is higher than in other systems and amplified abundant and reproducible band patterns.

Amplification reactions were performed in volume of 25 μL containing approximately 20 ng of template DNA, 15 ng of primer, 0.5 units of Taq polymerase (Promega), 0.1 μM of each dNTP, and 10 \times Buffer for Taq polymerase (Promega) as described by Zhou et al. (1998). RAPD primers were obtained from Shanghai Biotech (Shanghai, China). A total of 24 10-nucleotide-long primers were used. PCR amplifications were performed in a Perkin-Elmer DNA GeneAmp PCR System 9600, with an initial denaturation at 94°C for 4 min, followed by 38 cycles of 1 min at 94°C, 1 s at 39°C, 50 s at 72°C, and a final extension at 72°C for 7 min. Amplification products were separated by electrophoresis on 1% agarose gels. Sizes in kilo base (kb) pairs were inferred by comparisons with a Lambda DNA/ *Eco*RI + *Hind*III marker (Sino-America Bio). Gels were stained by ethidium bromide and recorded on a white/ultraviolet transilluminator with computer (UVP, Ultra-Violet Products).

RAPD Data Analysis

Twenty-two primers were found to produce well-amplified and reproducible electrophoretic band patterns. Using these informative bands, all individuals from every form were evaluated by RAPDistance 1.04 program in computer. For each individual, about 104 distinguishable fragments, including universal and specific, were scored with data coded as a vector of 1 or 0 representing band presence or absence respectively. Distance matrices among the 46 individuals of four forms were estimated based Apostol (1993). Phylogenetic trees showing the hierarchical structures of RAPD pattern affinities among individuals and forms were constructed from the distance matrices by NJTREE analysis, as implemented in the program of RAPDistance 1.04 (Apostol 1993).

RESULTS

Morphologic Difference Among the Four Forms

The data about morphologic traits of the four forms were summarized in Table 1. As shown in Table 1, their morphologic traits can be easily discriminated among the four different forms, especially in the aspects of exterior shell character and the ratio of shell height/shell breadth. Form Y is most analogous to typical parameters of *Atrina pectinata* described by Wang (1997).

Similarity and Heterogeneity of RAPD Band Patterns Within and Between the Four Forms

The similarity and heterogeneity of RAPD band patterns within and between the four forms were screened using 24 primers, of which, 22 primers reproducibly produced well-identifiable RAPD band patterns (1 to 12 bands ranging from about 0.2 to 2.5 kb; Table 2). No anyone of the RAPD band patterns produced by the 22 primers was identical in all of the four forms. As shown in Figure 3, there are significant differences in RAPD band patterns among the four forms. Some characteristic RAPD band patterns for the different form were produced, and only a few polymorphic bands were observed among individuals in each form. In comparison with form T and form S, forms G and Y exhibit more similarity in RAPD band patterns (Fig. 3).



Figure 2. Four sampling sites of pen shell in this study.

TABLE 1.

Observed growth of oyster populations at Horsehead.

Date	Temp (C)	Population #1		Population #2	
		n	Mean Length (mm)	n	Mean Length (mm)
10/15/1992	19.8	196	15.8		
11/11/1992	13.8	202	16.7	100	15.4
12/9/1982	7.6	140	16.2	79	16.4
1/28/1993	7	201	16.7	98	15.3
3/31/1993	12.4			175	14.1
4/14/1993		211	16.2		
5/3/1993	18.5	210	16.6	120	17.3
6/2/1993	22	239	16.7	94	17.9
6/28/1993	27.2	212	20.2	77	21.1
7/27/1993	28.8	208	25.3	76	26.6
8/24/1993	28.1	251	28.1	75	30.7
10/25/1993	19	251	33.2	75	36.8
11/16/1993	14.8	251	33.8	72	37.3
12/13/1993	8	243	33.9	72	37.6
4/4/1994	14	243	33.6	72	37
5/9/1994	18.5	235	33.2	72	36.7
6/10/1994	24.2	232	33.9	71	37.4
7/11/1994	28.6	234	37.1	70	41.4
8/8/1994	25.8	234	39.3	69	42.8
9/13/1994	24.5	230	41.4	69	43.9
10/18/1994	18	230	44.3	68	47.2
11/15/1994	17	230	45.3	68	49
2/20/1995	5	227	45.8	67	49
4/18/1995	16.9	229	45.4	65	49.1
5/24/1995	23.1	228	46.2	65	49.7
7/14/1995	28.9	160	47.2	38	50.7
8/29/1995	26.4	131	51	32	52.7
9/27/1995	20.7	135	52.2	30	52.9
10/17/1995	10.5	122	56.5	28	54.7

Detection of Specific RAPD Markers for Discriminating Different Forms

A total of 198 polymorphic fragments were scored from 22 primers (Table 2), and they are specific for one form, or shared by

TABLE 2.

Estimation of size specific growth rate incorporating temperature effects.

Month	Temp (C)	Estimated Monthly Growth Rate (mm/month) = (mx + c) where x is Mean Temperature		
		m	c	
J	4.8			
F	5.1			
M	7.9			
A	13.7			
M	19.0	year 0	0.27	-2.80
J	24.1	year 1	0.20	-1.74
J	27.0	year 2	0.17	-1.42
A	27.2	year 3	0.14	-1.16
S	24.6	year 4	0.11	-0.94
O	19.2	year 5	0.09	-0.77
N	13.7	year 6	0.07	-0.64
D	8.3			

two or three forms. Several primers, such as S451, S453, S463, S464, S470, S473, and S474, produced abundant band patterns and provided sufficient information for reliable discrimination of the four forms analyzed. As shown in Figure 3, primer S451 amplified one 2.2-kb fragment shared by all forms, one 0.5-kb fragment specific for form S, one 0.2 fragment specific for form T and another 0.8-kb fragment shared by forms G and Y. Primer S453 gave rise to two bands of 0.9 kb and 1.4 kb shared by forms G, Y and T, one 0.3-kb band shared by forms G and Y, and another 0.4-kb band specific for form S. Primer S463 generated four fragments in all individual of forms G and Y, two fragments in all form S individuals, five fragments in form T. Primer S470 yielded two fragments of 0.7-kb and 1.2-kb band specific for form S, three fragments of 0.2 kb, 0.8 kb and 1.0 kb specific for form T, and one 0.6-kb fragment shared by forms S, G and Y. Most of the polymorphic fragments can be used as specific RAPD markers for discriminating the four different forms in *Atrina pectinata*.

Average Genetic Distances and Phylogenetic Relationships

The average genetic distances and phylogenetic relationships were calculated and analyzed according to the total of 4788 distinguishable fragments obtained from the all RAPD band patterns. As shown in Table 3, the average genetic distances among individuals in form G (0.1362 ± 0.02487) and form Y (0.1195 ± 0.02622) are lower than that in form T (0.1769 ± 0.05324) and form S (0.1896 ± 0.02758), and the average genetic distance (0.1447 ± 0.02309) between form G and form Y is basically equal to that among individuals in the form G or Y. In contrast, the average genetic distances between form S and form T, between form S and form G, between form S and Y, between form T and form G, and between form T and form Y are very much high, and range from 0.4229 ± 0.02535 to 0.4888 ± 0.02227 (Table 3). These data indicate that the form G and form Y are closely related not only among individuals within the same form, but also between individuals from the two forms, and form T and form S are highly divergent.

The phylogenetic tree of the 46 individuals from the four forms was obtained by NJTREE analysis in the RAPDistance program, as shown in Figure 4. Three clusters were clearly distinguishable, in which two were corresponding to form S and form T respectively and one included forms G and Y. Any specimen was first clustered with other individuals from the same form. Apparently, forms G and Y are most closely related, while the most divergence occurs between forms S and G or Y. The constructed phylogenetic tree (Fig. 4) matches the average genetic distances among the four forms (Table 3).

DISCUSSION

In traditional mollusk taxonomy, characteristic shell features and organ structure are the main basis for categorizing different species and subspecies. But, some of these features are malleable and vary in accordance with environmental conditions. These various features and structures resulted in considerable confusion and controversy among taxonomists. For *Atrina pectinata*, debate as to whether the present species should be divided to two or more species or subspecies have lasted for years. In China, the four forms of pen shell were generally grouped to one species, *A. pectinata* (Wang 1997). In past decade, some new biochemical and molecular biologic methods, such as allozyme electrophoresis,

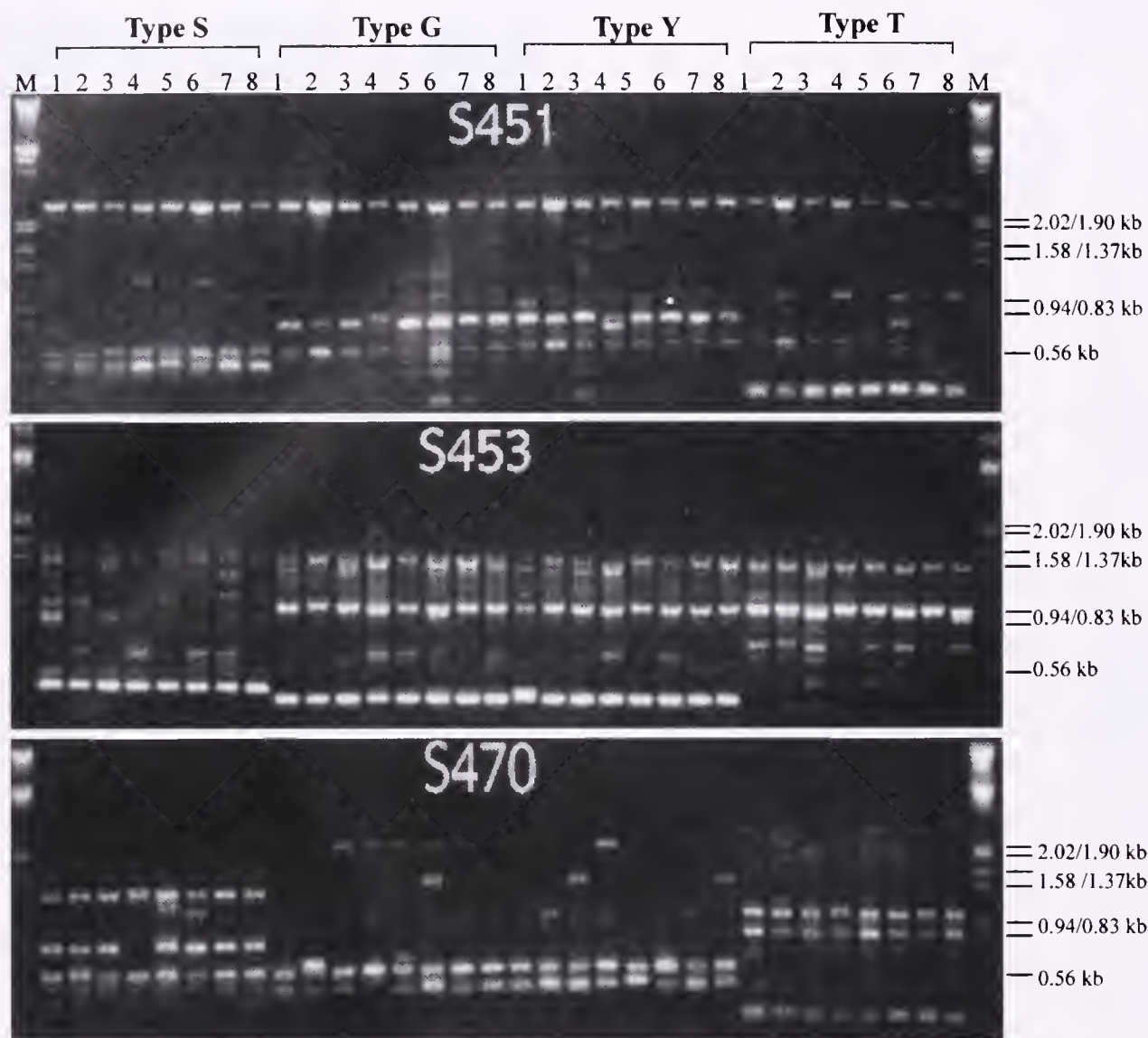


Figure 3. Electrophoretogram of three typical RAPD hand patterns produced by S451, S453, and S470 primers, indicating some specific or shared bands for one, two, or three forms.

mitochondrion DNA restriction mapping and RAPD polymorphic analysis, have been applied in comparison of genetic variation and taxonomic studies.

Thorp (1982) deduced from studies of classification of populations that the genetic similarity (I) below 0.85 between two populations should be on inter-species level, I value between 0.2 and

0.8 on level of inter-species in same genus, between 0.8 and 0.97 on level of populations of same species. In other words, while the genetic distance ($1 - I$) over 0.20 between two populations, the two populations should be categorized into different species.

In several mollusk species, the genetic distances (D) were estimated from data of isozyme electrophoresis and morphologic

TABLE 3.
Average genetic distances among the four forms of *Atrina pectinata*.

Form	G	Y	T	S
G	0.1362 ± 0.02487			
Y	0.1447 ± 0.02309	0.1195 ± 0.02622		
T	0.4319 ± 0.02469	0.4229 ± 0.02535	0.1769 ± 0.05324	
S	0.4888 ± 0.02227	0.4686 ± 0.02178	0.4458 ± 0.02881	0.1896 ± 0.02758

differences. Krause et al. (1994) surveyed the genetic variation among three geographic populations of calico scallops *Argopecten gibbus*. The genetic distance between populations was between 0.009 and 0.070. The nearer genetic distances were agreed with lower level of morphologic variation, both on the level of geographic population within same species. Three mussel species (*Mytilus edulis*, *M. galloprovincialis*, and *M. trossulus*) were ana-

lyzed by Skibinski et al. (1980), Grant and Cherry (1985), and Vainola and Hvilson (1991), the Nei's (1972) distance (D) among these three mussels was in the range of 0.16 to 0.28. Gosling (1992) regarded the above three mussels as a subspecies status. The swan mussel *Anodonta woodiana* living in same pond in Japan has been known to exhibit two forms. Tabe et al. (1994) made clear that D distance between the two forms was 0.707 and no

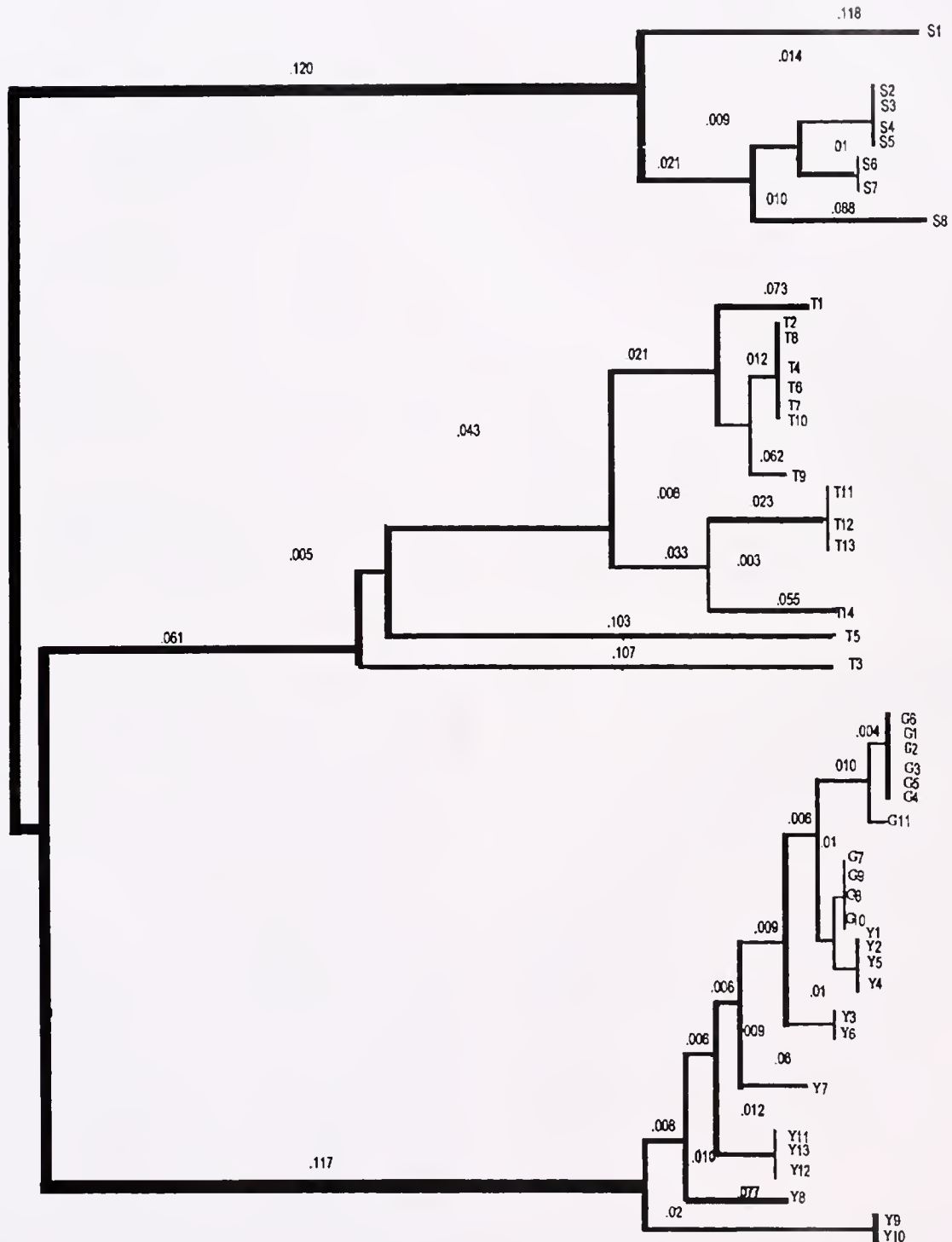


Figure 4. The phylogenetic trees of 46 individuals from four forms of pen shell by NJTREE analysis, as implemented by RAPD Distance, version 1.04

hybrid was observed, they suggested that these two forms should be regarded as distinct species. Buroker (1979a, 1979b) studied the intraspecific and interspecific genetic divergence of the family Ostreidae, and found that the D value of 0.0118–0.0408 was on the level of intraspecific populations, whereas above 0.165 was beyond sibling species. The reports on RAPD analysis in mollusc were not as much as that of isozyme electrophoresis. Patwary (1994) first used RAPD technique in bivalve mollusc for analyzing genetic divergence in scallop *Placopecten magellanicus* Gmelin and distinguished different allele frequencies in different populations. Wilding et al. (1998) analyzed genetic differentiation of morphotypes of periwinkle *Littorina saxatilis* from two locations of England. They considered the diverse RAPD patterns as evidence of separate gene pools. Liu et al. (1998a, 1998b) analyzed RAPD polymorphism of oyster species in northern China, and found that genetic distances between three species *Crassostrea talienwhanensis*, *C. plicatula* and *C. gigas* were among 0.333–0.400, whereas the intraspecific distances between four geographic populations of *C. talienwhanensis* were below 0.2004. These researches were almost conformed to inferences about D distance suggested by Thorp (1982).

In Pinnadea species, very few data about their genetic background could be obtained. Koji Yokogawa (1996) had examined and calculated genetic divergence of nonscaly and scaly pen shell *Atrina pectinata* distributing in partial Japanese marine region by isozyme electrophoresis. Based on the high D value (0.469) and low frequency of hybrids, Koji Yokogawa (1996) suggested that the two forms would be taxonomically distinguished. In this study, we demonstrated that different forms of *Atrina pectinata* amplified diverse RAPD band patterns, and that specific markers created by some primers can be efficiently used to discriminate the four different forms of pen shell. Twenty-two primers produced a total of

4,788 distinguishable bands from 46 individuals of the four forms, and 87 markers specific for some forms (Table 2). The dendrogram and average genetic distances clearly indicate phylogenetic relationships among the four forms. Among the four different forms, form G was clustered together with form Y, and their average genetic distance is the smallest (≤ 0.15), even smaller than the intra-form distance of form T (≥ 0.17) and S (≥ 0.18). The most divergence occurred between form S and forms G (or Y), their genetic distance is more than 0.48. The data imply that form G has a very high genetic homogeneity to form Y, and form S is distinctly contrasted to form G (or Y) in the genetic background. It should be reliable to say that the pen shell, previously regarded as one species, *Atrina pectinata*, should be a conspecific complex composed of at least two species. Form G and Y are same species, and form S is another species different from them. Form T remains undecided, and it might belong to either group or be the third species or subspecies.

Of course, RAPD data in our study could only be a proof for classification of these four forms, further investigations should be conducted to provide more and convincing evidences. Two experiments are going to be carried out: 1) by crossing between the form G and form S, the most genetically distant forms, to observe the survival status of their hybrids, 2) by cloning and sequencing specific RAPD fragments to develop diagnostic PCR SCAR primers (Zhou et al. 2001) for reliable discrimination of the four forms. These new evidences will be benefit to further reclassification of *A. pectinata*.

ACKNOWLEDGMENTS

These studies were supported by the Chinese Academy of Sciences (KSCX2-SW-303) and the State Key Laboratory of Freshwater Ecology and Biotechnology.

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REPRODUCTIVE PATTERN OF THE BLOOD ARK, *ANADARA OVALIS* FROM THE NORTHEAST COAST OF FLORIDA

ALAN J. POWER,¹ JOSE NUNEZ,² MARCY MITCHELL,¹ RANDAL L. WALKER¹ AND LESLIE STURMER²

¹Shellfish Research Laboratory, University of Georgia Marine Extension Service, Savannah, Georgia 31411-1011; ²Whitney Laboratory, University of Florida, St. Augustine, Florida 32080

ABSTRACT In blood arks, the sexes are separate; however, a low incidence of hermaphrodites was observed in the current study (2.17%). Males occurred more frequently than females (m/f = 2.68). Ripe arks were present year round outside of the period from August to November 2002. A bimodal reproductive pattern was apparent with a peak in gonadal development during the late spring-early summer months (45% ripe in May), followed by a quiescent period in the late summer-fall, and followed in turn by a minor peak during the winter months (21% ripe in December). Spawning was apparent in all months with the exception of September, and individuals in this phase were most numerous in June (50%) and July (64.29%). Dribble spawning is likely used as a strategy to extend its spawning period and increase its reproductive success. A low incidence (3.33%) of parasitic infection by digenetic trematodes resulting in castration was also noted. The implications of these findings on the aquaculture potential of this species are discussed.

KEY WORDS: ark, gametogenesis, reproduction, sex ratio, spawning, *Anadara ovalis*

INTRODUCTION

Several ark species (*Scapharca*, *Arca*, and *Anadara* spp.) form the basis of economically significant molluscan fisheries and extensive culture operations (Baquero et al. 1982, Baquero 1989, Broom 1985, MacKenzie 2001, Manzi & Castagna 1989, Nie 1990, Umezawa 1992). A member of the ark shell family Arcidae, the blood ark, *Anadara ovalis* (Bruguière 1789) occurs from Cape Cod, Massachusetts, to the West Indies and Brazil, at depths ranging from the low-tide line to >3 m (Abbott 1974, Anderson et al. 1984, Rehder 1981, Walker & Gates 2001). The species favors sandy deposits (Alexander 1993) and salinities above 15 ppt (Chanley & Andrews 1971). With a short lifespan (<5 y), early maturity (10–12 mm/–8 mo), rapid growth rates, and established markets in the Northeast, this bivalve has shown tremendous potential as an aquaculture species to diversify hard clam operations in Southeastern coastal waters (McGraw et al. 1996, McGraw et al. 1998, Power & Walker 2001, Power & Walker 2002, Walker 1998).

In Florida, *A. ovalis* occurs mainly on the eastern coast whereas the related ponderous ark (*Noetia ponderosa*, Say 1822) also inhabits the Gulf Coast. A concerted effort is currently underway among researchers and extension agents on both coasts of Florida and on the Georgia coast to investigate the hatchery-rearing protocol for both species. A systematic histologic examination of the gonads is a critical step providing information on the gametogenic cycle and sex ratios. By knowing the natural gametogenic cycle of a species, hatchery operators will know when to collect brood stock in the field while they are at their optimum ripe stage prior to their spawning. Histologic analysis of gonadal tissue also allows hatchery operators to know if animals from the natural population can be spawned more than once per year. This can save time and money in conditioning the bivalves to an optimum ripe stage within the hatchery. Knowledge of the sex ratio of a species determines the number of individuals that must be held for conditioning and spawning.

The reproductive biology of the blood ark has not previously been investigated for populations anywhere in Florida. In Virginia, blood arks are reported to spawn in the summer months when water temperatures reach above 17°C (Chanley & Andrews 1971, McGraw et al. 1998). In Georgia, blood arks spawn earlier, from late spring through summer (Power & Walker, 2002). In

general, spawning periods and gametogenesis in marine bivalves start earlier and last longer in southern geographical areas than in northern ones (Eversole 1989, Thompson et al. 1996), and therefore the gametogenic cycles from Florida can be expected to be unique and clearly warrant investigation.

MATERIALS AND METHODS

Blood arks were collected from harvested clam bags during processing at certified shellfish wholesalers (Sturmer et al. 1995) and subsequently replanted in a commercial clam lease on the Matanzas River near "Marineland" south of St. Augustine, Florida, for holding and continued growout over the annual study period. The salinity and temperature of the river were monitored at the Whitney Laboratory floating dock every 30 min using a YSI 6600 data sonde.

Each month between May 2002 and April 2003, approximately 30 specimens were shipped to the Shellfish Research Laboratory in Savannah, Georgia. Immediately upon arrival, each individual was measured for shell length, width, height, and total weight (McGraw et al. 1996), and a mid-lateral gonadal sample (ca. 1 cm²) was dissected for histologic analysis. Notes on the gonad color were recorded. Gonadal tissue was fixed in Davidson's solution, refrigerated for 48 h, washed with 50% ethanol, and preserved in 70% ethanol until processing. We processed tissues according to procedures outlined in Howard and Smith (1983). The examination of prepared gonadal slides was conducted with a Zeiss Standard 20 microscope (20×). Each animal was sexed and assigned to a developmental stage as described by Walker and Heffernan (1994) and Spruck et al. (1994). A staging criteria of 0 to 5 was used for early active (EA = 3), late active (LA = 4), ripe (R = 5), partially spawned (PS = 2), spent (SP = 1), and inactive (IA = 0). The determination of monthly gonadal index (G.I.) values was obtained by averaging the number of specimens ascribed to each category score. We tested sex ratios against a 1:1 ratio with chi-square (χ^2) statistics (Elliott 1977).

RESULTS

Monthly mean water temperature and salinity data for the Matanzas River are shown in Figure 1. Water temperatures ranged from 12 to 28°C between the winter and summer months. Salinities showed less of an annual pattern, and values ranged from 22

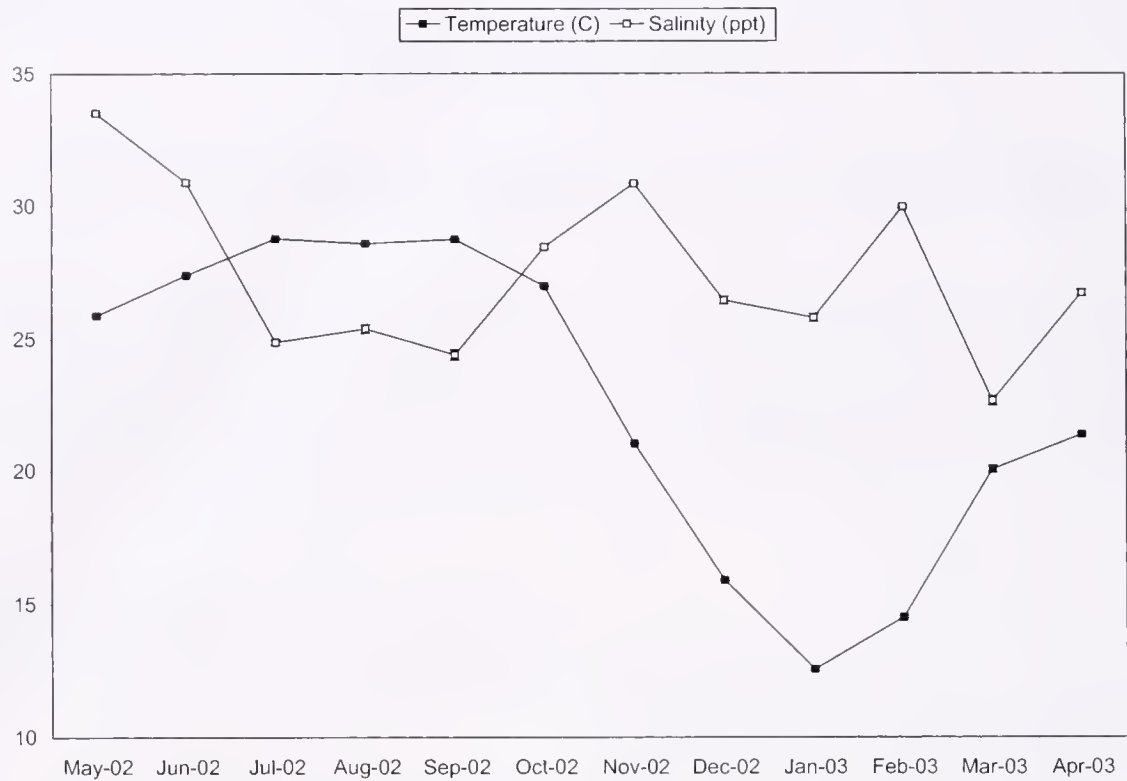


Figure 1. The mean monthly water temperature and salinity of the Matanzas River, Florida, from May 2002 to April 2003.

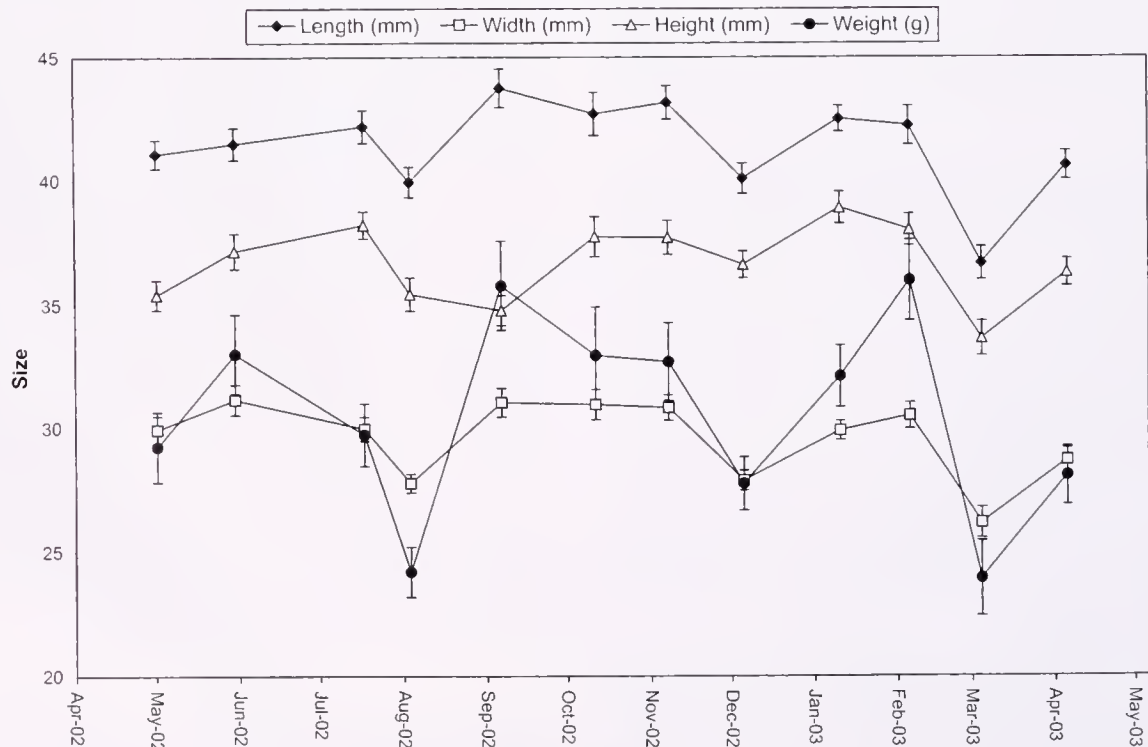


Figure 2. The mean monthly shell length, shell width, shell height (mm \pm standard error) and total wet weight (g \pm standard error) of the blood arks, *Anadara ovalis*, sacrificed for the histologic analysis between May 2002 and April 2003.

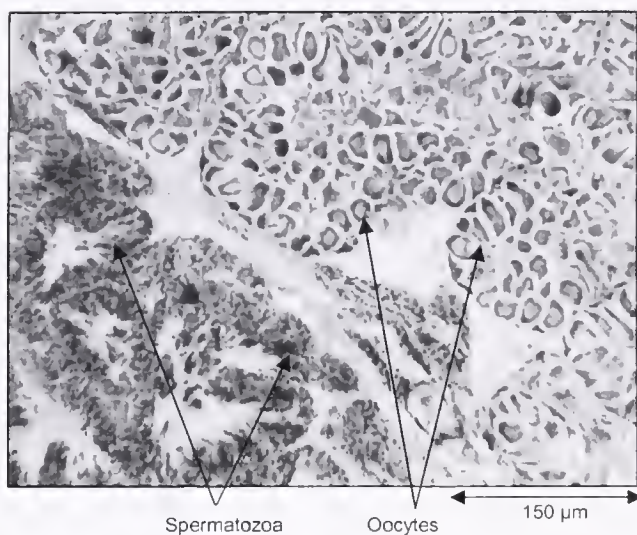


Figure 3. A histologic section through the gonad of a hermaphroditic blood ark, *A. ovalis*, showing ripe oocytes and spermatozoa.

to 33 ppt. Figure 2 displays the mean size parameters (length, width, height, and weight) of monthly samples. The average length of blood arks from all samples combined was 41.34 mm (± 0.22 SE). The largest and smallest ark observed in terms of shell length, width, height, and weight measured 52.59 mm, 39.58 mm, 41.15 mm, 58.90 g, and 29.50 mm, 21.00 mm, 88.10 mm, and 13.10 g, respectively.

Of the 323 arks examined histologically, 73 (22.60%) were sexually indeterminate, 177 (54.80%) were male, 66 (20.43%)

were female, and 7 (2.17%) were hermaphroditic (Fig. 3). Figure 4 reveals the size distribution of arks according to these categories. Both male and female arks were most abundant in the 42-mm shell-length class and represented in similar proportions in the smaller and larger size classes. Hermaphroditic arks occurred between 38 and 48 mm. Males dominated in all monthly samples with the exception of September 2002, where there were two with four females; however, a large percentage (75%) of individuals in this sample did not display any gonadal activity and therefore were sexually indeterminate (Fig. 5). The χ^2 test revealed that the overall male/female ratio of 2.68 was significantly different from parity ($\chi^2 = 7.89$; $P < 0.01$). Histologic examination and visual observations of the gonads revealed that all orange-red colored gonads were females whereas those showing white coloration were typically males.

Ripe arks were present in most samples, with the exception of those taken from August to November 2002 (Fig. 6). Similarly, partially spawned individuals were observed throughout the year with the notable exception of September 2002, when all gonads were completely spent. While an extended spawning period from May to September was observed, a bimodal annual pattern was apparent in the G.I. plot. Gonadal development peaked during the late spring-early summer months (45% ripe in May), followed by a quiescent period in the late summer-fall, and followed in turn by a minor peak during the winter months (21% ripe in December). The largest coordinated spawning effort was in June and July, where 50% and 64.2% of individuals had partially spawned gonads. Partially spawned gonads often had smaller areas of ripe and late active follicles.

A low incidence (3.33%) of parasitic infection by digenetic trematodes resulting in castration was also noted. No seasonal

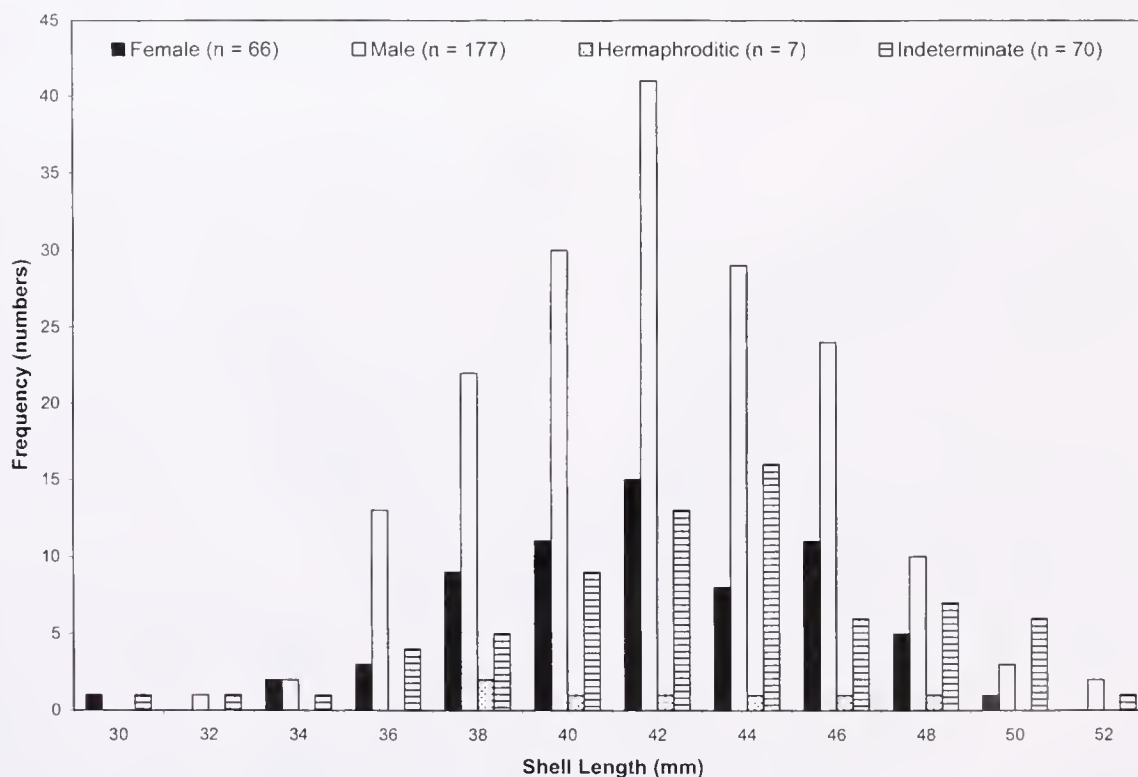


Figure 4. The frequency size distribution (2-mm shell length classes) of blood arks, *A. ovalis*, identified as male, female, hermaphrodite, or indeterminate during the course of the annual study.

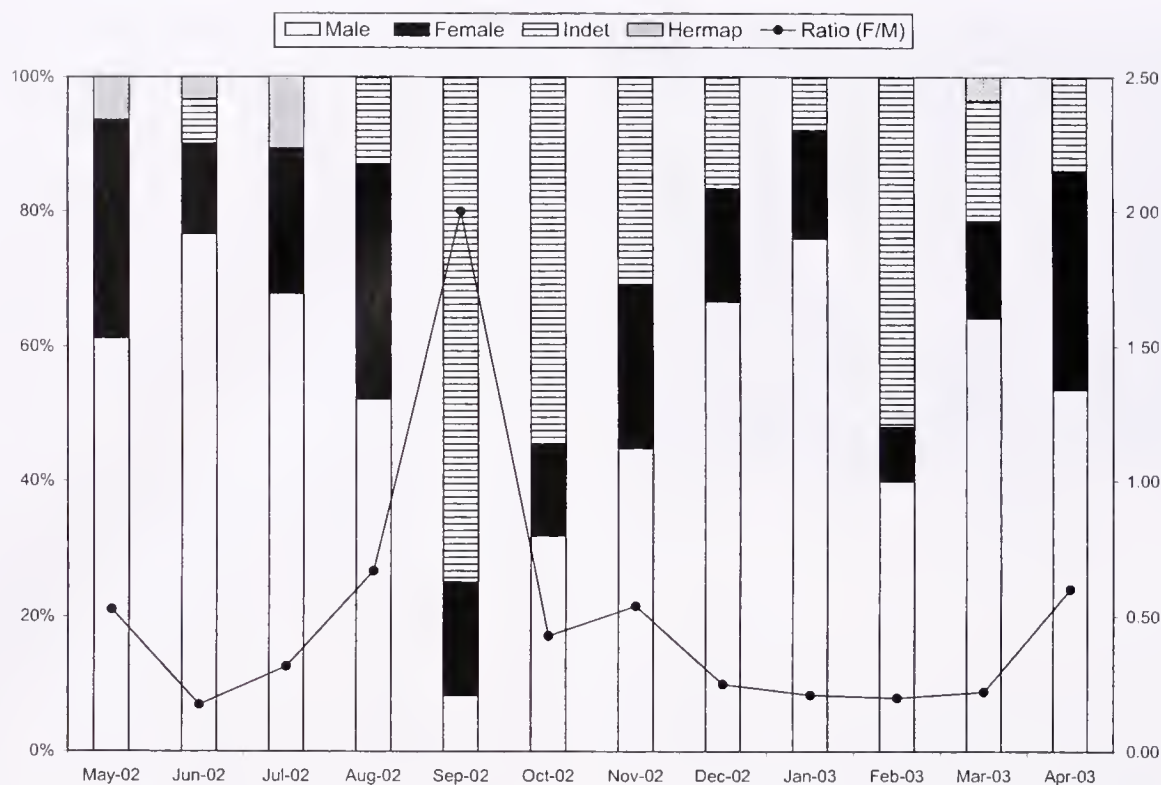


Figure 5. Variations in the female:male ratio of blood arks, *A. ovalis*, in monthly samples taken from St. Augustine, Florida, between May 2002 and April 2003. Shown also are the percentage of individuals each month labeled as male, female, hermaphrodite, and indeterminate.

pattern in parasitic infection was evident, with one specimen being infected in the months of May, June, July, September, November, December 2001, and March 2002, and two specimens in the months of January and February 2002. Spearman's rank correlation coefficients detected a significant correlation between mean water salinity levels and the mean monthly G.I. ($r_s = 0.67$, $t = 2.07$, $df = 11$, $\alpha = 0.05$).

DISCUSSION

In Long Island Sound, New York, the transverse ark, *Anadara transversa* (Say, 1822), is reported to spawn between May and August (Loosanoff & Davis 1963). Further south in the Chesapeake, this species spawns from May to September (Chanley & Andrews 1971). In Georgia, a more extended period is observed for the transverse ark with major spawning occurring between April and July, and some spawning also occurring in the winter months of December and January (Walker & Power, 2003). Similarly, for the related blood arks, a spawning period from May to September has been identified for the Chesapeake area (McGraw et al. 1998) whereas a more extended period over most of the year but peaking in the summer has been reported for Georgia (Power & Walker 2002). Further south in Costa Rica, ripe *Anadara tuberculosa* (Sowerby, 1883) are present throughout the year with a peak in spawning activity between May and September (Cruz 1984a). Additionally, *Anadara similis* (Adams, 1852) and *Anadara grandis* (Broderip & Sowerby, 1829) from these waters are also ripe year round but have peaks during the winter months (Cruz 1984b, Cruz 1987). From this synopsis, it would appear that the spawning pattern of the Arcidae family becomes less confined to a single narrow season with decreasing latitude and the associ-

ated reduction in temporal fluctuations in temperature and coastal phytoplankton abundance. Based on the current study, it is apparent that blood arks from the northeast of Florida can also remain in the ripe and spawning phases for most of the year and that salinity may be a controlling factor in the gametogenic cycle.

A prolonged spawning period has distinct advantages and disadvantages for producing the species in a commercial hatchery. During the microscopic analysis of histologic slides, partially spawned gonads with patches of ripe and late active follicles were frequently observed. It is therefore likely that this species achieves a long spawning season by maturing different sections of its gonad at different times. This would give rise to a "dribble" spawning reproductive strategy, wherein gametes are not released synchronously as a massive event within the population, but instead small amounts are periodically released over an extended time period. In a hatchery, such a strategy would reduce the number of viable gametes available through a single induced spawning effort. As a result, either more individuals or repeated spawning attempts during several weeks and months might be necessary to generate sufficient numbers of larvae.

A clear advantage is that broodstock can be obtained and spawned almost anytime outside of the fall months, allowing for multiple spawnings per year. Of course, the optimal time for spawning is the early summer based on the percentage of individuals synchronously in the desirable late active, ripe, and partially spent gonadal stages (>80%). During the winter reproductive peak, less than 50% of individuals display gonads at these stages. Therefore, spawning in the early summer months would reduce the number of individuals required for conditioning.

A reduction in the number of broodstock required is important

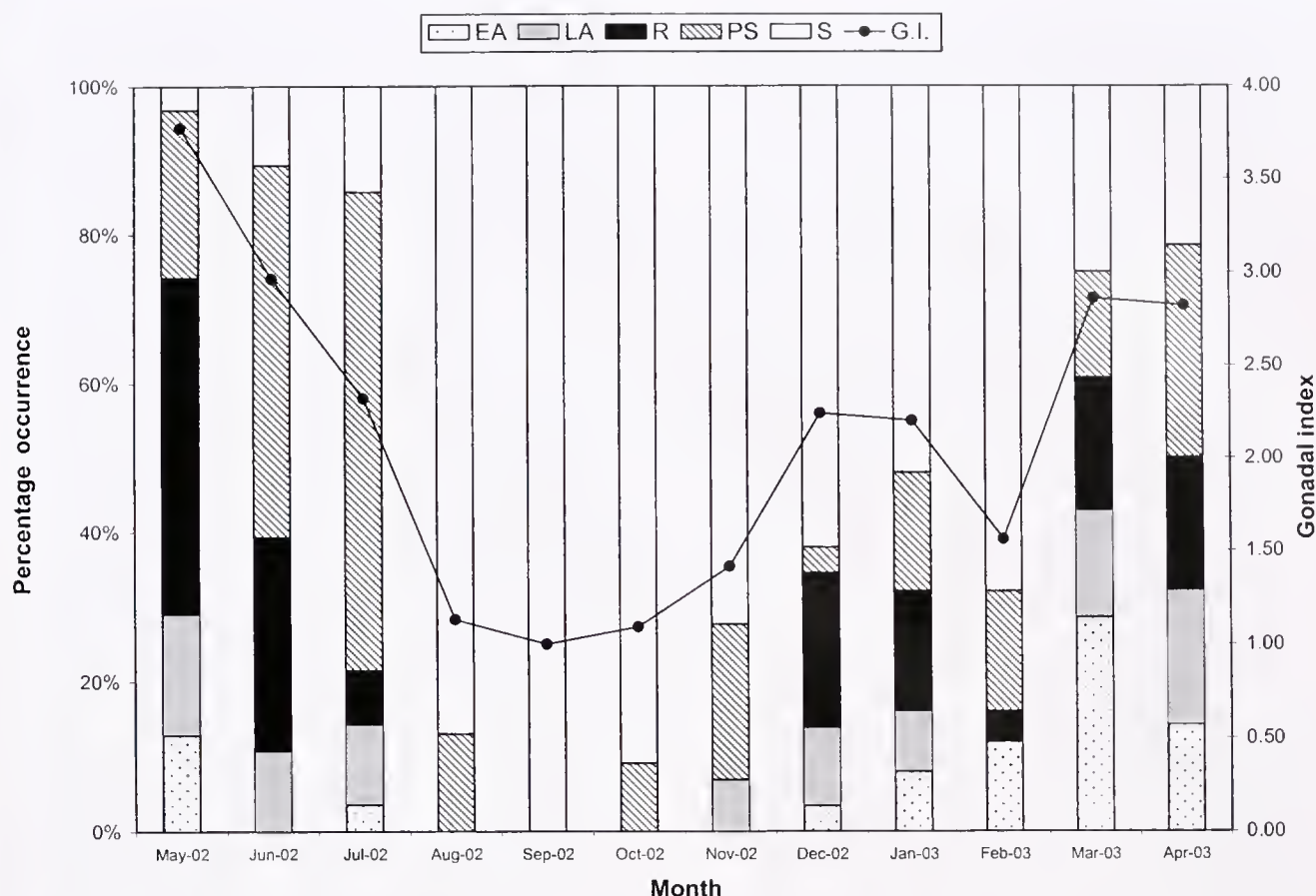


Figure 6. The relative frequency (percentage) of each gonadal developmental phase (EA = early active, LA = late active, R = ripe, PS = partially spawned, and S = spent) of *A. ovalis* between May 2002 and April 2003. Monthly gonadal index (G.I.) values are also shown and were determined by averaging the number of specimens ascribed to each category score (EA = 3, LA = 4, R = 5, PS = 2, SP = 1).

because only one in three to four blood arks are likely to be female (M:F = 2.68:1.00). The male domination reported here is in general agreement with previously reported sex ratios for the species in Virginia (1.98:1.00 M:F; McGraw et al. 1998) and in Georgia (2.44:1.00 M:F; Power & Walker 2002). This is, however, the highest male to female ratio recorded for a member of the Arcidae family (see review in Power & Walker 2002). Hermaphroditism was observed for the species in the current study for the first time, albeit at a low incidence. One individual displayed a large area of mature oocytes sandwiched between two similarly sized areas of spermatozoa whereas the other six had only a few follicles of the extra gamete. Hermaphroditism is rare in the whole Arcidae family. A low incidence has been reported for *Anadara granosa* (0.003%) and *Anadara senilis* (0.004%) Broom (1983, 1985, respectively). It has been suggested that these might be protandric hermaphrodites. Males typically dominate protandric bivalve species in the first year whereas older age classes are generally equal. However, the specimens examined in the current study were generally of the larger and older size classes and were still dominated by males.

Males and females were also represented in similar ratios throughout the entire shell length size distribution analyzed (i.e., 30–52 mm). In addition, one male ark maintained in a conditioning tank with 39 others (both sexes) at the Whitney Laboratory was induced to spawn female gametes 2 mo after having last released spermatozoa. This does not support a protandric hermaphroditic life cycle, and the observed phenomenon cannot be explained.

ACKNOWLEDGMENTS

This work was supported by congressional funds from the State of Florida (Grant No. USDA Grant # 2002-34453-11946). The authors thank Mr. Phil Cubbedge for providing and holding the arks on his lease and Mr. Micah Alo for his assistance in the field. Thanks to Ms. Rebecca Green, Ms. Dodie Thompson, and Ms. Mary Sweeney-Reeves for dissecting the samples and to Ms. Lisa Calvo and Prof. Gene Bureson at the Virginia Institute of Marine Science Department of Environmental and Aquatic Animal Health for diagnosing the trematode infections.

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HISTOLOGICAL STUDIES ON HERMAPHRODITISM, GAMETOGENESIS AND CYCLIC CHANGES IN THE STRUCTURES OF MARSUPIAL GILLS OF THE INTRODUCED ASIATIC CLAM, *CORBICULA FLUMINEA*, AND THE KOREAN CLAM, *CORBICULA LEANA*

GAB-MAN PARK^{1,*} AND EE-YUNG CHUNG²

¹Department of Parasitology, Kwandong University College of Medicine, Gangneung, Gangwon-do 210-701, Korea; ²School of Marine Life Science, College of Marine Science and Technology, Kunsan National University, Kunsan, Chollabuk-do 573-701, Korea

ABSTRACT The marsh clams, *Corbicula fluminea* and *Corbicula leana*, are functional hermaphrodites. They usually appear to be surrounded by numerous spermatozoa in the hermaphroditic follicles. In both species, the follicular ganglia (consisting of the neuronal fiber and neuronal soma-like cells at its periphery) are associated with neurosecretion and the differentiation of complex innervated nerve structures during spermatogenesis and are widely distributed in the follicles in the ripe and spawning stage. *Corbicula fluminea* and *C. leana* have two pairs of gills, with the inner-demibranchs acting mainly as marsupia. The non-marsupial demibranchs are not separated, but in the marsupial demibranchs, cyclic changes in the structures of the inner-demibranchs of the gills appear, with the depletion of ripe eggs during incubatory periods and the production of mature and ripe eggs during nonincubatory periods. The reproduction of triploid *C. fluminea* and *C. leana* may occur by parthenogenesis without self-fertilization (or cross-fertilization) by eggs and sperm. The DNA contents of the somatic (gill) and gamete (spermatozoa) cells of *C. fluminea* are the same. Because reproduction is parthenogenetic, numerous spermatozoa may participate in the activation of the mature eggs and egg cleavage, as a stimulus only for parthenogenesis in the same hermaphroditic follicle or the gonophore.

KEY WORDS: *Corbicula fluminea*, *Corbicula leana*, gametogenesis, hermaphroditism, Korea, North American

INTRODUCTION

The freshwater clams *Corbicula fluminea* and *Corbicula leana* are hermaphrodites that brood their larvae in the inner-demibranchs (Britton and Morton 1982, Miyazaki 1936). Okamoto and Arimoto (1986) suggested that *C. leana* reproduce by gynogenesis. *Corbicula fluminea* and *C. leana*, on the basis of chromosomal and karyological studies have been reported as being triploid (Okamoto and Arimoto 1986, Park et al. 2000). The triploid condition may closely be related to hermaphroditism.

The Korean clam, *C. leana*, is one of the commercially important edible clams. However, the North American *C. fluminea* was introduced from Asia in the 1900s and is now widely distributed throughout the United States (Britton and Morton 1982, McMahon 1982), where it has become a biofouling pest (Matice 1979, McMahon 1977). There have been a few previous studies of the reproduction of the *Corbicula* species (Kennedy and van Huekelem 1985, Kraemer 1978, Kraemer 1984, Kraemer et al. 1986, Morton 1982, Williams and McMahon 1986). In addition, gametogenesis and the corresponding morphologic changes in the inner-demibranchs have not been examined. Finally, there is the question whether the reproduction of the two triploid *Corbicula* species involves self-fertilization, cross-fertilization, or parthenogenesis.

The purposes of this study were to understand functional hermaphroditism, gametogenesis, the cyclic changes in the structures of the inner-demibranchs, and the duration of the pediveligers released from the parent clams. The DNA content of the spermatozoa and gill tissues of *C. fluminea* were also compared.

MATERIALS AND METHODS

Among *Corbicula* species used for the current study, a total of 366 North American *C. fluminea* specimens collected from the Bethel Lake Dam, Pitman, Gloucester County, New Jersey, between July 1995 and December 1996 were examined shortly after

being identified. Three hundred fifty-two specimens of *C. leana* were collected from the Keum River, Korea, between February 2000 and December 2001. The 30 to 40 individuals were collected on a monthly basis for this study.

The live clams were transported to the laboratory of the Museum of Zoology, University of Michigan, and the Department of Parasitology, Kwandong University, and the shell lengths and heights measured using Vernier calipers and their total weight determined using a balance. Histologic preparations were made to study the sexuality, gametogenesis, and morphologic changes in the structures of the demibranchs by light microscopy. The visceral mass and the gill tissues were subjected to standard histologic procedures (dehydrated in alcohol and embedded in paraffin), and 5–7- μ m sections prepared using a rotary microtome. The sections were then mounted onto glass slides, stained with Hansen's hematoxylin-0.5% eosin and Mallory's triple stain, and observed using light microscopy.

To compare the relative DNA content of the spermatozoa and gill tissue of *C. fluminea*, cells were isolated on a glass slide by cutting small pieces of gonad and gill tissues in distilled water and air-drying it before fixing with 70% ethanol. The spermatozoa and gill cells from one individual were placed on the slide. The cells were stained with DNA-specific dyes PI (propidium iodide) and DAPI (4',6-diamidino-2-phenylindole), and the relative DNA content (fluorescence intensity) per cell estimated by microfluorometry, as in Komaru et al. (1988). The DNA content was assayed at least three times at each of three different concentrations of spermatozoa and gill cells. Twelve individuals were used for these assays. The spermatozoa could easily be distinguished from the other spermatogenic cells due to their elongate and curved morphology.

RESULTS

Sexuality and Functional Hermaphroditism

The gonad of the freshwater clams *C. fluminea* and *C. leana* is located between the digestive diverticula and the outer fibromus-

*Corresponding author. E-mail: gmpark@kwandong.ac.kr

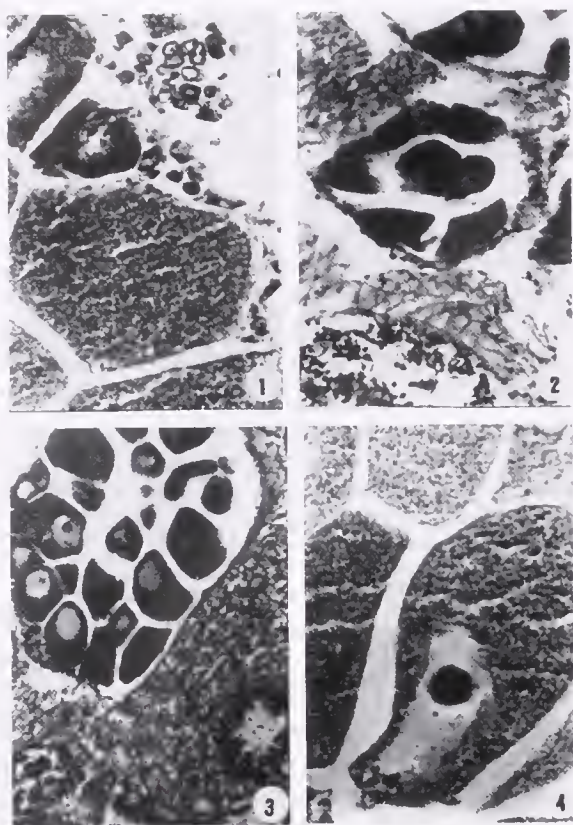


Figure 1. *Corbicula fluminea*: Photomicrograph of oogonia in the follicular walls showing undifferentiated mesenchymal tissues and eosinophilic cells.

Figure 2. *Corbicula fluminea*: Photomicrograph of previtellogenic oocyte, with eosinophilic nucleoli in the nucleus.

Figure 3. *Corbicula fluminea*: Photomicrograph of late vitellogenic oocytes, with oogenic follicles and hermaphroditic follicle.

Figure 4. *Corbicula fluminea*: Photomicrograph of a mature oocyte, with a large nucleolus in the nucleus and numerous yolk granules in the cytoplasm.

cular layers, which are compacted by the fibrous connective tissues and muscle fibers. As the gonad matures, it extends to the lowest part of the muscular layers, around the foot. Both *Corbicula* species were hermaphrodites (monoecious), and gonad consisted of a number of oogenic, spermatogenic, or hermaphroditic follicles. Both female and male germ cells were present in the oogenic, spermatogenic, or hermaphroditic follicles (Figs. 1–20). More specifically, triploid *C. fluminea* and *C. leana* showed hermaphroditism, and a group of the ovotestis (Figs. 6, 16, and 20), or intrafollicular embryos, usually appeared to be surrounded by numerous spermatozoa in the hermaphroditic follicles during egg cleavage (Figs. 8 and 20). The functional hermaphroditism was shown in the hermaphroditic follicles.

Gametogenesis

The oogenesis of the two *Corbicula* species occurred in the oogenic or hermaphroditic follicles between the digestive and the outer fibromuscular layers and was divided into five stages: oogonial, previtellogenic oocyte, vitellogenic oocyte, and mature oocyte stages. Also, spermatogenesis occurred in the spermatogenic or hermaphroditic follicles, between the digestive diverticula and the outer fibromuscular layers, and was divided into five

stages: spermatogonial, primary spermatocyte, secondary spermatocyte, spermatid, and spermatozoon stages. The characteristics of gametogenesis of the two *Corbicula* species are as follows.

Corbicula fluminea

This species is a hermaphrodite. The gonad consisted of a number of oogenic (Figs. 1, 2, 3, and 4), spermatogenic (Figs. 2, 3, 5, and 7), or hermaphroditic (Figs. 6 and 8) follicles.

Oogenesis Oogenesis occurred in the oogenic and hermaphroditic follicles. The oogenic and hermaphroditic follicles located near the outer muscular layer begin to develop toward the visceral mass. A number of oogonia appeared along the follicular walls (16–18 μm in diameter) and had a round nucleus containing a nucleolus in its center. One nucleolus in the nucleus was distinct in appearance, though the cytoplasm of the oogonium was very poor stained. At this time, a number of undifferentiated mesenchymal tissues and eosinophilic cells were both located near the follicle walls (Fig. 1). The oogonium developed into the previtellogenic oocyte. The previtellogenic oocyte (30–36 μm in diameter) had a round nucleus containing one or more small eosinophilic nucleoli along the nuclear envelope, and the cytoplasm begin to grow in volume. Undifferentiated mesenchymal and eosinophilic granular cells were abundant on the follicular wall. There were widely distributed interfollicular connective tissues near the follicles (Fig. 2). Early vitellogenic oocytes grew to 130–140 μm in diameter and became eosinophilic oval or pentagonal oocytes in the oogenic or hermaphroditic follicles (Fig. 3). Mature oocytes (about 150–170 μm) had one large nucleolus, 3–4 small nucleoli in the nucleus, and numerous yolk granules in the cytoplasm. At this stage, the vitelline envelope of the mature oocyte was surrounded with a gelatinous substance (Fig. 4).

Spermatogenesis Spermatogenesis occurred between the digestive diverticula and the outer fibromuscular layers. The spermatogonia were 8–9 μm in diameter and contained a large oval nucleus located in the wall of the spermatogenic or hermaphroditic follicles. Undifferentiated mesenchymal cells and eosinophilic cells were located near the spermatogonia and spermatocytes (Fig. 5). The spermatocytes developed into the spermatids. At this time, the spermatids in the center of lumina of the hermaphroditic follicles were occupied with a few oocytes; the interfollicular connective tissues were also widely occupied (Fig. 6). At the early differentiation stage of the spermatid, the shape of the nucleus changed gradually and became slightly elongated and narrow. After spermatogenesis (transformation of the spermatid into the spermatozoon), a number of the spermatozoa formed needle-shaped sperm clusters in the male follicles (Fig. 7). The spermatozoon was approximately 12–13 μm in length, and the sperm head was approximately 1.0 μm in width.

In the partially spawned follicle, an embryo uncleaving, surrounded by a number of sperm, appeared in the hermaphroditic follicle (Figs. 8 and 9), indicating that *C. fluminea* is a functional hermaphrodite. Male reproductive tissue was less common than female tissue. At this time, the gonoduct was located near the spermatogenic follicle, and the follicular ganglia (consisting of the neuronal fiber and neuronal soma-like cells at its periphery), associated with neurosecretion and the differentiation of complex innervated nerve structures during spermatogenesis, were widely distributed in the follicles during the ripe and spawning stages (Figs. 10 and 11).

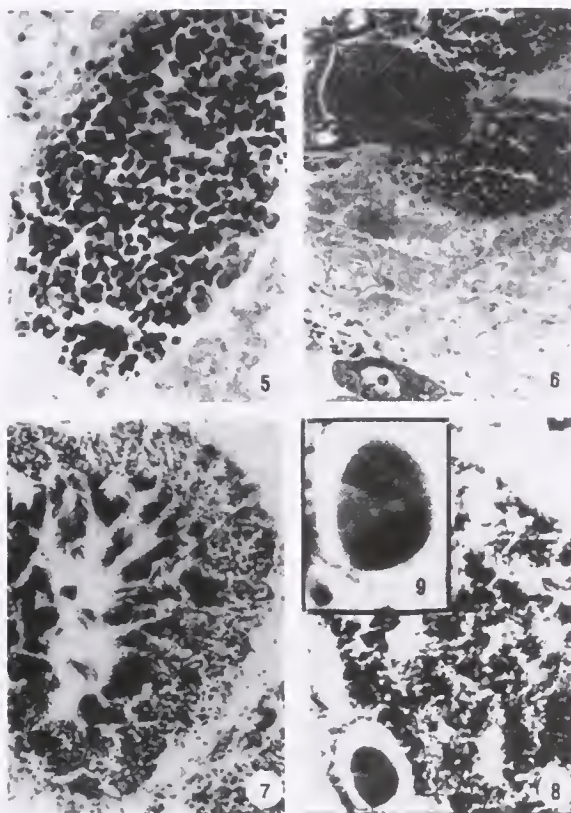


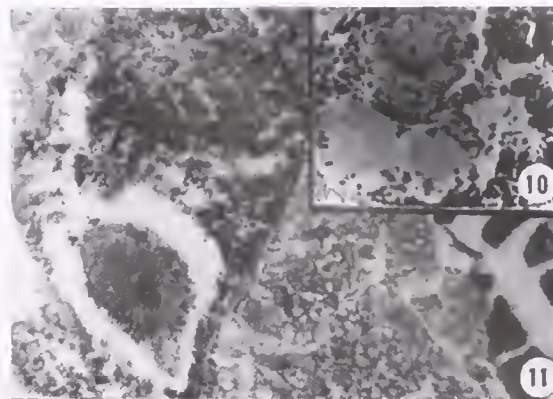
Figure 5. *Corbicula fluminea*: Photomicrograph of spermatozoa in the follicular wall, with undifferentiated mesenchymal cells and eosinophilic cells in follicle.

Figure 6. *Corbicula fluminea*: Photomicrograph of hermaphroditic follicles, with spermatocytes and spermatid in the lumen and interfollicular connective tissues.

Figure 7. *Corbicula fluminea*: Photomicrograph of testis in the mature stage, showing the needle-shaped sperm clusters in the spermatogenic follicles.

Figures 8 and 9. *Corbicula fluminea*: Photomicrograph of an embryo during egg cleavage, surrounded by a number of sperms in the hermaphroditic follicle.

Cyclic Changes in Structures of the Inner-Demibranchs A pair of the inner-demibranchs acted mainly as marsupium. Incubated ripe eggs and D-shaped veliger larvae were rarely found within a pair of the outer-demibranchs. The non-marsupial demibranchs were not separated and had no secondary septa and tripartite marsupial structure. In this case, the outer-membranes of the demibranchs were slightly thicker (Fig. 12A). In the case of the marsupial demibranchs, the morphologic and structural changes in the inner-demibranchs of the gills showed a distinct seasonal alternation or periodicity in relation to depletion of ripe eggs during the incubatory periods, with the production of mature and ripe eggs during the nonincubatory periods. In the late nonincubatory period, during gametogenesis, or before depletion of the ripe gametes, the tripartite marsupial structures completely disappeared, and the epithelial cells lining the inner-lamellar spaces of the inner-demibranchs became gradually thicker (Fig. 12B). During the early incubatory period of the inner-demibranchs (removal of ripe gametes or early developing embryos from the gametogenic follicles to the gills), a number of ripe eggs and D-shaped veliger larvae were filled in thickened inter-lamellar spaces of the inner-demibranchs (Fig. 12C). In the late incubatory period (gonads in



Figures 10 and 11. *Corbicula fluminea*: "Follicular ganglion" section from anterior region of visceral mass. Many neuronal-like soma(s) at the periphery of the "ganglion" and its location within a well-developed spermatogenic follicle.

the partially spawned stage), a number of incubated D-shaped veliger or pediveliger larvae were found in the inter-lamellar spaces of the inner-demibranchs. Thereafter, as the marsupial demibranchs began to be separated by the secondary septa, the secondary septa produce secondary water tubes (Fig. 12D). Consequently, in the early nonincubatory period, the tripartite marsupial organization that was formed by the secondary septa and water tubes appeared in the inner-demibranchs (Fig. 12E). At this time, the inter-lamellar spaces of the tripartite marsupial demibranchs were vacant, as a number of the D-shaped veligers in the marsupial gills were released.

Some characteristics of the relationships between the structural changes of the inner-demibranchs in the marsupial gills and gametogenesis in the gonads were found: most gonads became degenerate and appeared to be depleted of female and male gametes during the incubatory and larval release periods. Therefore, inhibition of gametogenesis might occur during the incubatory periods (especially during the mid-summer and early autumn incubation and D-shaped veliger larvae release).

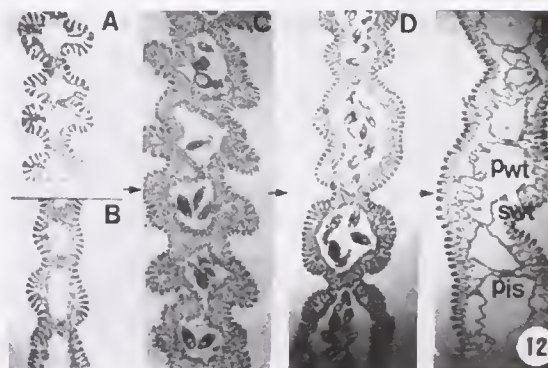


Figure 12. *Corbicula fluminea*: Photomicrographs of cyclic changes in structures of the inner-demibranch during gametogenic cycle. (A) Cross section of the outer-demibranch, (B) cross section of the inner-demibranch, (C) a number of ripe eggs and D-shaped veliger larvae fill the thick inter-lamellar spaces of the inner-demibranch, (D) marsupial demibranch shown separated by the secondary septa, (E) in the early nonincubatory period, the tripartite marsupial organization is formed by the secondary septa and secondary water tube. (Abbreviations: pis, primary interlamellar septum; pwt, primary water tube; swt, secondary water tube.)

Corbicula leana

This species is a triploid (Okamoto and Arimoto 1986) and a hermaphrodite. The gonad consisted of a number of the oogenic (Figs. 13 and 15) and spermatogenic (Figs. 17, 18, and 19) or hermaphroditic (Fig. 20) follicles. The spermatogenic and oogenic or hermaphroditic follicles were distributed among the interfollicular connective tissues. The characteristics of gametogenesis of this species were as follows.

Oogenesis Oogenesis occurred in the oogenic follicles. Many oogonia (15–18 μm) propagated along the follicle wall near the mesenchymal tissues. The oogonia had one large basophilic nucleus and light basophilic cytoplasm (Fig. 13). The oogonia developed into the previtellogenic oocytes; at the beginning of cytoplasmic growth, each oocyte (ranging in diameter from 20 to 40 μm) had an egg-stalk and was attached to the follicle walls of the oogenic follicle near the spermatogenic follicle, which contained a number of spermatids. In the early vitellogenic oocytes (50–70 μm), the cytoplasm was markedly stained with eosin (Fig. 14). The vitellogenic oocytes (80–100 μm) and the late vitellogenic oocyte (110–130 μm) were located in the center of the lumina of the follicle, and numerous eosinophilic yolk granules in the cytoplasm were filled (Fig. 15). The mature oocytes (more than 140–160 μm) had 4–7 nucleoli in the nucleus, and many yolk

granules in the cytoplasm were filled. The mature oocytes were surrounded by a gelatinous substance. Several mature oocytes were found in the lumen of the hermaphroditic follicles and contained numerous spermatozoa (Fig. 16).

Spermatogenesis The testes were composed of a number of spermatogenic and hermaphroditic follicles, including oogenic tissues. The spermatogonia, which were distributed along the follicular wall, were approximately 9 μm in diameter and had relatively little cytoplasm. The spermatogonia developed into the primary spermatocytes, and the primary spermatocytes developed into the secondary spermatocytes. The chromatin in the nucleus of the spermatocyte became gradually more concentrated, while the volume of the cytoplasm of the secondary spermatocyte gradually became smaller. The secondary spermatocytes developed into spermatids, which were darkly stained with hematoxylin, and distributed in the center of the lumina of the spermatogenic follicle. Stratified layers of the spermatogonia, spermatocytes, and spermatids formed in the male follicle (Fig. 17). The spermatids developed into spermatozoa in the spermatogenic or hermaphroditic follicles, containing a few ovotestis on the oogenic tissue (Fig. 18). A number of needle-shaped sperm clusters, formed by numerous spermatozoa, filled the spermatid follicle. The spermatozoon was approximately 12 μm in length, and the head was approximately 1.2 μm in width. During spermatogenesis, follicular ganglia (consisting of neuronal fiber and neuronal soma-like cells) were widely distributed in the spermatogenic follicle, but gradually disappeared (Fig. 19). In the spawning period, the embryo-like body, surrounded by a number of sperm clusters, appeared in the hermaphroditic follicle (Fig. 20).

Relative DNA Content of Spermatozoa and Somatic (Gill) Cells

In *C. fluminea*, the relative content of the DNA in the spermatozoa was identical to that in the gill cells (Fig. 21). Komaru et al. (1997) reported that *C. leana* is a triploidy species, and from microfluorometric analyses, the sperm and somatic cells were shown to have the same DNA content. They suggested that meiosis I or II may be abortive in spermatogenesis, with only one equal division resulting in nonreductional spermatozoa.

DISCUSSION

In general, hermaphroditism is seen more frequently in freshwater than marine mollusks (Van der Schalie 1970). *Corbicula* species can be categorized into three major groups, based on their reproductive characters and ecologies (Miyazaki 1936). Species belonging to Group 1 are monoecious, viviparous, and incubatory. They have nonswimming planktonic veliger larvae and live in freshwater. The species belonging to Group 2 are dioecious, oviparous, nonincubatory, and also live in freshwater regions. The species belonging to Group 3 are dioecious and oviparous. They do not incubate their young, have free-swimming planktotrophic larvae, and live in brackish waters. In this study, *C. fluminea* and *C. leana* were confirmed to be hermaphroditic species (Group 1). According to our histologic observation, the two *Corbicula* species were hermaphroditic throughout their lifetime, with no sex reversal. According to Coe's report (Coe 1943), cited by Heard (1975), hermaphroditic conditions in pelecypods were divided into four categories, according to the sequence of reproductive events: (1) functional hermaphroditism (eggs and sperm produced simultaneously), which can be subdivided into two groups: (i) normal

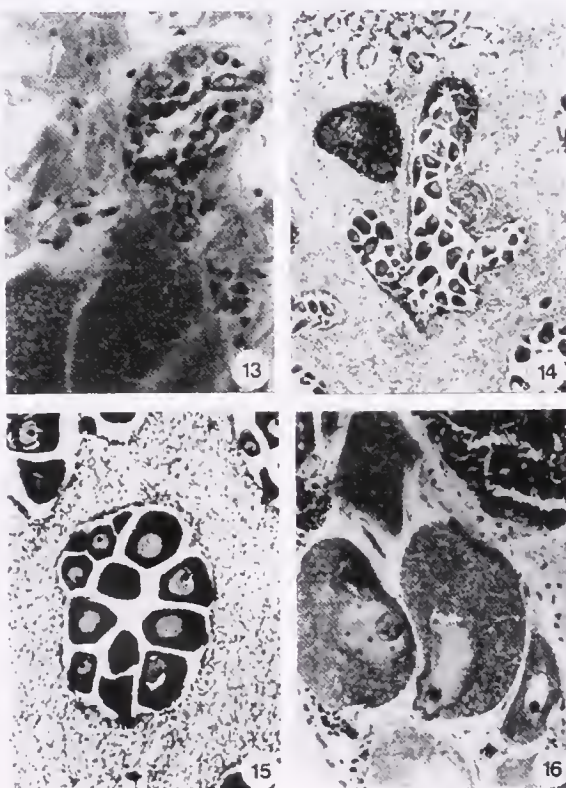


Figure 13. *Corbicula leana*: Photomicrograph of oogonia in the follicular walls, showing undifferentiated mesenchymal tissues and eosinophilic cells.

Figure 14. *Corbicula leana*: Photomicrograph of early vitellogenic oocytes, with large basophilic nucleolus in the nucleus.

Figure 15. *Corbicula leana*: Photomicrograph of the vitellogenic oocytes, with the late vitellogenic oocytes located in lumen of the follicles.

Figure 16. *Corbicula leana*: Photomicrograph of the mature oocytes.

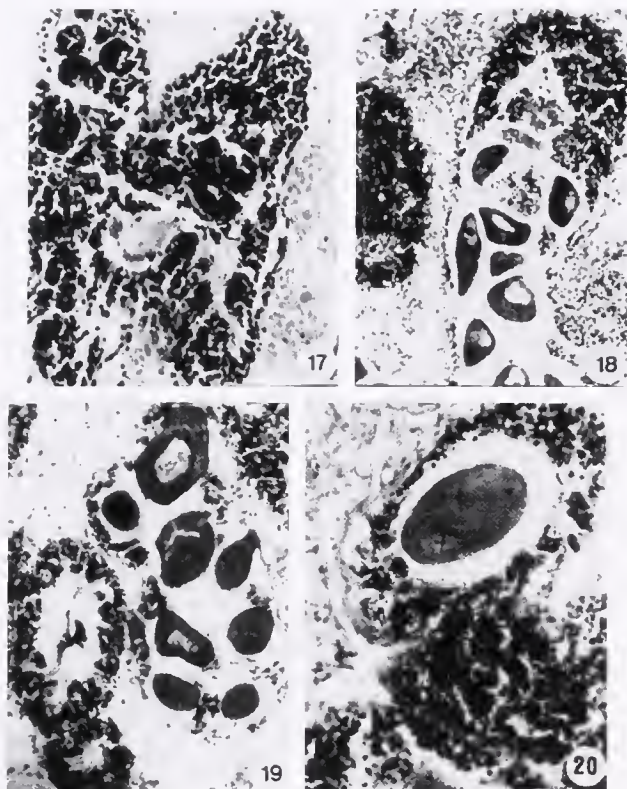


Figure 17. *Corbicula leana*: Photomicrograph of spermatozoa in the spermatogenic follicle.

Figure 18. *Corbicula leana*: Photomicrograph of hermaphroditic follicles, with spermatozoa in the spermatogenic follicles.

Figure 19. *Corbicula leana*: Photomicrograph of follicular ganglia.

Figure 20. *Corbicula leana*: Photomicrograph of the spawning period, with the embryo-like body surrounded by a number of sperm clusters in the hermaphroditic follicle.

(typically in monoecious species) and (ii) accidental or development (typically in dioecious species); (2) consecutive sexuality (single sex-reversal, usually protandrous); (3) rhythmical sexuality (>1 sex-reversal, usually protandrous); and (4) alternative sexuality (adults function seasonally as separate sexes). Monoecious species can be grouped as male (predominance of testicular tissue; animal not gravid) and female (ovarian tissue slightly or greatly exceeding quantity of testicular tissue; animal may become gravid) hermaphrodites. In the current study, *C. leana* and *C. fluminea* were found to be functional hermaphrodites.

Kraemer and Lott (1977) reported that although the largest *Corbicula* species exceeded 20 mm in size, they consistently showed a clear predominance of ovarian over testicular development. *Corbicula fluminea* had greater volumes of oogenic than spermatogenic tissue. The oogenic follicles were larger, with a clear predominance and well-developed oocytes. In particular, in the case of triploid species, this phenomenon might occur because of parthenogenetic reproduction. The results of this study on *Corbicula* species support the previous findings by Kraemer and Lott (1977). Okamoto and Arimoto (1986) proposed that triploid species might closely be related to hermaphroditism. With reference to hermaphroditism and fertilization, there have been some reports that *C. fluminea* carry out both self-fertilization (Kraemer 1978, Kraemer et al. 1986) and cross-fertilization (Kraemer 1978). In this study, some intrafollicular embryos were found in the course

of egg cleavage in the hermaphroditic follicles of *C. fluminea* (Fig. 8). At a glance, some intermingling of a number of spermatozoa and a few oocytes in the same follicle suggest that self-fertilization may occur in the hermaphroditic follicle. However *C. fluminea* is a triploid species (Park et al. 2000), and its DNA content in the nucleus of the somatic cell (gill) is the same as that in the gamete cell (sperm). Komaru et al. (1997) reported that *C. fluminea* was a diploid species and produced nonreductional spermatozoa. If *C. fluminea* sexually reproduces itself, the DNA content in the nucleus of the somatic cell, and the number of the homozygote genes, would increase in volume and chromosome numbers, respectively, due to egg and sperm fertilization. Therefore, reproduction of this species may occur by parthenogenesis, without self- or cross-fertilization of eggs and sperm. The DNA contents in the nuclei of the somatic and gamete cells of *C. fluminea* were the same. In the case of parthenogenesis, it is assumed that numerous spermatozoa may participate in activation of the mature eggs, and the eggs cleavage as a stimulus only for parthenogenesis in the same hermaphroditic follicle or the gonopore. Komaru et al. (1998) reported that cytologic observations and DNA microfluorometry of the hermaphrodite freshwater triploid clam *C. leana* revealed androgenetic development.

Bivalves have two pairs of gills. In the case of *Corbicula* species, two pairs of gills act as the marsupia, but the pair of inner-demibranchs mainly play an important role as the marsupia; in general, a pair of the outer-demibranchs occasionally act as the marsupia. Differences in the structural and morphologic changes between the non-marsupial and marsupial demibranchs are very clear. Heard (1975) described that in *Anodonta*, the non-marsupial demibranchs did not form the secondary septa, and a comparatively large number of filaments were distributed between the inner membranes of the primary septa, whereas marsupial demibranchs were divided by the secondary septa that produced the secondary water tubes, resulting in tripartite marsupial organization. However, Kwon and Park (1985) stated that after the glochidia had been released, the secondary water tube was continuously present in *Lanceolaria acrorhyncha*. In this study, the patterns reported by Heard (1975) were found in *C. fluminea*. In the late nonincubatory period, during gametogenesis or before spawning, the structures of the tripartite marsupial inner-

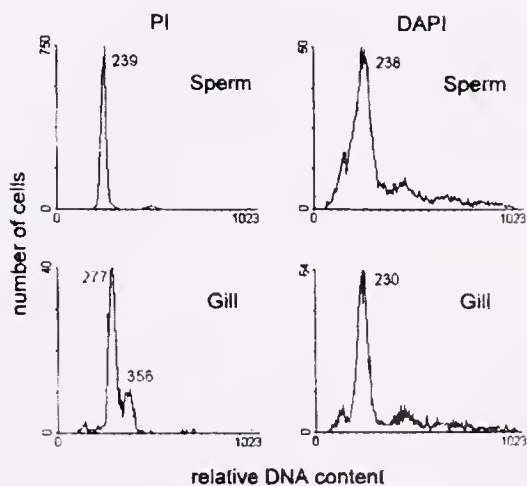


Figure 21. *Corbicula leana*: Fluorescence histograms of the spermatozoa and gill cells of *C. fluminea*, stained with PI (propidium iodide) and DAPI (4',6-diamidino-2-phenylindole dihydrochloride).

demibranchs disappeared, while the inner-membranes of the marsupial gills were vacant and became very thick. In the early incubatory period, during movement of ripe gametes or early developing embryos in the gametogenic follicles to inner-demibranchs, a number of ripe eggs or D-shaped veliger larvae filled the thickened epithelial cells lining the inter-lamellar spaces of the inner-demibranchs. In the late incubatory period, the late pediveligers or straight-hinged juveniles (approximately 230–240 μm in size), which had grown in the marsupial gills for approximately 7–10 days, began to shed into the environmental water. At this time, the primary water tubes began to separate, and the secondary water septa formed in the marsupial gills. Consequently, in the early nonincubatory period, the tripartite marsupial organization appeared in the inner-demibranchs of the gills. In our histologic study, the two *Corbicula* species were found to be hermaphrodites, with specified demibranchs of the gills. Particularly, gametogen-

esis inhibition occurred in the gametogenic follicles during the gills incubatory period whereas active gametogenesis occurred during the nonincubatory period. The structural hypertrophy of the epithelial cells lining the inter-lamellar spaces was especially changed in relation to the nonincubatory and incubatory periods of the inner-demibranchs. Therefore, the results of this study suggest that the larvae may receive nourishment from the hypertrophied epithelial cells that line the inter-lamellar spaces of the inner-demibranchs of the adult clam. Thus, the morphologic changes in the structure of the marsupial gills, during the incubatory and nonincubatory periods, with gametogenesis and spawning patterns, may occur cyclically or have a certain short-term periodicity. In conclusion, *C. fluminea* and *C. leana* are functional hermaphrodites, with the spermatozoa in the triploidy *Corbicula* species showing an identical DNA content to the somatic tissue of the parent.

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FEEDING BY LARVAE OF THE MUSSEL *MYTILUS GALLOPROVINCIALIS* ON RED-TIDE DINOFLAGELLATES

HAE JIN JEONG,¹* JAE YOON SONG,² CHANG HOON LEE,³ AND SEONG TAEK KIM²

¹School of Earth and Environmental Science, College of Natural Sciences, Seoul National University, Seoul 151-747; ²Department of Oceanography, College of Ocean Science and Technology, Kunsan National University, Kunsan 573-701; ³Red Tide Research Center, Kunsan National University, Kunsan 573-701, Republic of Korea

ABSTRACT To investigate feeding by the larvae of the mussel *Mytilus galloprovincialis* on red-tide dinoflagellates, we measured grazing rates of *M. galloprovincialis* larvae as a function of larval age and prey concentration when feeding on several species of the red-tide dinoflagellates *Alexandrium affine*, *Cochlodinium polykrikoides*, *Lingulodinium polyedrum*, *Prorocentrum minimum*, *Prorocentrum nicans*, and *Scrippsiella trochoidea*, as well as the flagellate *Isochrysis galbana* as a control species. The larvae were able to ingest all dinoflagellates offered in the current study; however, first feeding of the larvae on each species of the dinoflagellates occurred 9–13 days after fertilization, whereas that for *I. galbana* occurred after 5 days. Ingestion rates of the larvae on unialgal diets of the dinoflagellates and *I. galbana* increased with increasing larval age up to 17–21 days, but were saturated or showed a continuous increase thereafter. Ingestion rates of 25-day-old larvae feeding on unialgal diets of the dinoflagellates increased rapidly with increasing prey concentration up to 1000–2200 ng C mL⁻¹, but were saturated at higher prey concentrations. The harmful alga *C. polykrikoides*, which has been responsible for great losses in the aquaculture industry, was the optimal prey. Maximum ingestion and clearance rates of the larvae on these dinoflagellates were 14–69 ng C predator⁻¹ day⁻¹ and 1.5–11.4 μ L predator⁻¹ h⁻¹, respectively. *M. galloprovincialis* larvae, one component of microzooplankters, exhibited higher maximum ingestion and clearance rates than previously reported for other microzooplankters, such as *Fragilidium* cf. *mexicanum* (mixotrophic dinoflagellate), *Protoperdinium* cf. *divergens*, *Polykrikos kofoidii* (heterotrophic dinoflagellates), or *Tiarina fusus* (small ciliate), but lower rates than *Strombidinopsis* sp. and *Favella* sp. (large ciliates). The results of the current study suggest that dinoflagellates sometimes can be primary prey for the *Mytilus* larvae, and the grazers compete with other microzooplankters for dinoflagellate prey. Also, red-tide dinoflagellates can be used for culturing the *Mytilus* larvae as prey in the aquaculture industry.

KEY WORDS: benthic–pelagic interaction, benthos, bivalve; HAB, mollusca, plankton, *Mytilus*

INTRODUCTION

Bivalves and dinoflagellates are major components of benthos and plankton in marine environments, respectively (Ruppert & Barnes 1994, Steidinger & Tangen 1997). Red tides and/or harmful algal blooms dominated by phototrophic dinoflagellates often have caused large-scale mortality of adult bivalves (e.g., ECOHAB 1995). As a consequence, there have been many studies on interactions between adult bivalves and red-tide dinoflagellates (Widdows et al. 1979, Nielsen & Stromgren 1991, Shumway & Cembella 1993, Shumway et al. 1997, Matsuyama et al. 1997, Bricelj & Shumway 1998). Bivalve larvae spend a certain period after hatching as plankton and need to feed on planktonic prey. Red-tide dinoflagellates often dominate phytoplankton assemblages in coastal waters. Thus, there is a high possibility that bivalve larvae frequently encounter red-tide dinoflagellates. While there are some studies on the grazing by bivalve larvae on microflagellates and/or diatoms in the laboratory (Bayne 1965, Riisgård et al. 1980, Sprung 1984a, Sprung 1984b, Leonardos & Lucas 2000), there are a few studies on the interactions between bivalve larvae and red-tide dinoflagellates (Wikfors & Smolowitz 1995, Matsuyama et al. 2001); no data are available for bivalve larvae grazing rates as a function of red-tide dinoflagellate concentration and first feeding age for prey species.

Among bivalves, the genus *Mytilus* has a cosmopolitan distribution (e.g., Seed 1976). Some species are commercially important and cultivated at high densities in many countries (Hickman 1992). *Mytilus galloprovincialis* is a common bivalve in Europe (Moroño et al. 1998, Tubaro et al. 1998), Asia (Matsuyama et al. 1997,

NFRDI 1999), and Oceania (Gardner 2002) and is spreading to other areas as an invasive species (McQuaid & Phillips 2000).

To investigate interactions between bivalve larvae and red-tide dinoflagellates, we established cultures of *M. galloprovincialis* larvae and conducted experiments to examine their functional response when fed a variety of red-tide dinoflagellates. Our goal was to explore the predator–prey relationship between *M. galloprovincialis* larvae and red-tide dinoflagellates by determining the larval ingestion and clearance rates as functions of prey concentration and larval age.

The maximum ingestion and clearance rates of *M. galloprovincialis* larvae on unialgal diets of red-tide dinoflagellates were compared with those of other microzooplankters (heterotrophic dinoflagellates, and ciliates), which also are potential competitors, when feeding on the same prey species. Results of the current study provide a basis for understanding the interactions between bivalve larvae and red-tide dinoflagellates.

MATERIALS AND METHODS

Culture of Phytoplankton Prey

The dinoflagellates (Table 1) which have formed red tides in many coastal waters (Eppley & Harrison 1975, Jeong 1995, Ismael 2003) were grown at 19°C in enriched 1/2 seawater media (Guillard & Ryther 1962) without silicate, under continuous illumination of 100 μ E m⁻² s⁻¹ provided by cool white fluorescent light. Only cultures in the exponential growth phase detected by cell count were used for the feeding experiments. Carbon contents for red-tide dinoflagellates were estimated from the volume of cells in batch cultures according to Strathmann (Strathmann 1967).

*Corresponding author. E-mail: hjeong@snu.ac.kr

TABLE 1.

Species of red-tide dinoflagellate prey and a flagellate *Isochrysis galbana* used in the current study, listed in order of cell volume.*

Species	ESD + Standard Error (μm)	Approximate Volume (μm^3)
<i>Isochrysis galbana</i> (IGKC99)	4.8 ± 0.2	57
<i>Prorocentrum minimum</i> (PMJH99)	12.9 ± 3.6	1100
<i>Cochlodinium polykrikoides</i> (CPKS00)	23.2 ± 3.1	6600
<i>Alexandrium affine</i> (AAJM00)	24.0 ± 1.1	7200
<i>Scrippsiella trochoidea</i> (STKP99)	25.1 ± 2.8	8300
<i>Prorocentrum micans</i> (PMJH99)	26.0 ± 2.3	9200
<i>Lingulodinium polyedrum</i> (LPSD95)	37.9 ± 4.5	28,500

* Mean equivalent spherical diameter (ESD) was measured by the PAMAS-SVSS particle counter. Volume was calculated according to the equation: Volume = $4/3 \pi (\text{ESD}/2)^3$. The number of cells measured, n , was >2000.

Preparation of *M. galloprovincialis* Larvae

Approximately 300 adults of the blue mussel *M. galloprovincialis* were collected from an aquafarm off Yeosu, Korea, in March 2001 when the seawater temperature was 12°C and the salinity was 33.4 psu. The shell length of the mussels ranged from 45 to 65 mm and gonads of most individuals were at late active or ripe stages. The mussels were transported to the laboratory within 6 h after collection and then acclimated to an experimental temperature (15°C) for two months. During acclimation, the mussels were maintained in two 200-L aquariums where seawater freshly filtered through 5 μm GF/F filters and air from an air pump were supplied. The microflagellate *Isochrysis galbana* (final concentration = approximately 10^3 cells mL^{-1}) was provided as prey every day. The mussels were observed daily to check their condition, and dead mussels were removed immediately after they were found.

Spawning of the mussels was induced from May to June when natural spawning usually occurs in the Korean coastal waters. Approximately 10 individuals were used for each spawning period. Before spawning, the shell surface of the mussels was scraped to remove epibionts and rinsed with freshly filtered seawater. To

induce spawning, the mussels were exposed to air for 1 h, put back into a 10-L aquarium filled with freshly filtered seawater, and then the water temperature was increased gradually to 25°C . Most of these mussels released sperm and eggs within 30 min after the water temperature reached 25°C . As soon as any male first released sperm, the other males were removed to avoid possible polyspermy. One hour after spawning, all mussels were removed, and then aliquots of the water in the aquarium were taken to determine the fertilization rate. During the current study, we induced spawning 10 times, and the fertilization rates were always >95%. The mean diameter of the fertilized eggs was approximately 60 μm . The egg suspension was passed through a 100- μm mesh screen to remove fecal material and other large particles, and a 35- μm mesh screen was used to collect fertilized eggs without excessive numbers of sperm and smaller eggs. The eggs were rinsed three times with 5- μm filtered and autoclaved seawater and were then incubated in a 20-L aquarium at 15°C in darkness without aeration. After 1-day incubation, most eggs had developed to the trochophore larval stage. From the first day after fertilization, *Isochrysis galbana* (final concentration = approximately 5×10^3 cells mL^{-1}) was provided to larvae as prey every day. Incubation water was wholly renewed every day.

Ingestion Rates as a Function of Larval Age

Experiments 1 to 7 were designed to measure ingestion and clearance rates of *M. galloprovincialis* larvae as a function of the larval age (elapsed time after fertilization), when feeding on unialgal diets of 6 red-tide dinoflagellate species and *Isochrysis galbana* as a control species (Table 2). Feeding experiments were conducted when larvae were 1, 5, 9, 13, 17, 21, and 25 days old, because settlement of the larvae had started when they were 27 days old.

One day before these experiments were conducted, cultures of *M. galloprovincialis* larvae unfed (for the 1-day-old larvae) or growing on *Isochrysis galbana* (for the other aged larvae) were sieved through meshes of 80–150 μm , and the larvae retained were transferred into 1-L polycarbonate (PC) bottles. The bottles were filled to capacity with filtered seawater and placed on plankton wheels rotating at 0.9 rpm and incubated at 15°C under continuous

TABLE 2.

Experimental design: Values in prey and predator columns represent actual initial densities (cells mL^{-1} for prey and individuals mL^{-1} for predator) followed by calculated carbon biomass (ng C mL^{-1}) in parentheses.

Experiment No.	Prey		Predator
	Species	Density	Density
1	<i>Isochrysis galbana</i>	72,100–84,067 (865–1009)	3–7
2	<i>Prorocentrum minimum</i>	7227–9976 (1084–1496)	4–9
3	<i>Cochlodinium polykrikoides</i>	1532–1883 (1072–1318)	3–9
4	<i>Alexandrium affine</i>	1103–1560 (838–1185)	4–9
5	<i>Scrippsiella trochoidea</i>	1245–1667 (1058–1417)	3–8
6	<i>Prorocentrum micans</i>	947–1363 (890–1223)	3–5
7	<i>Lingulodinium polyedrum</i>	439–582 (1098–1455)	4–9
8	<i>Prorocentrum minimum</i>	255 (38), 723 (108), 1443 (216), 7638 (1146), 15483 (2322), 32288 (4843)	1–5
9	<i>Cochlodinium polykrikoides</i>	27 (19), 151 (106), 298 (208), 1492 (1044), 3120 (2184), 4147 (5925)	1–5
10	<i>Alexandrium affine</i>	33 (25), 130 (99), 266 (202), 1354 (1029), 2178 (1655), 4423 (3362)	1–7
11	<i>Scrippsiella trochoidea</i>	20 (17), 113 (96), 224 (191), 1126 (957), 2433 (2068), 4930 (4191)	1–7
12	<i>Prorocentrum micans</i>	24 (23), 108 (102), 236 (221), 1159 (1089), 2249 (2114), 4703 (4420)	1–4
13	<i>Lingulodinium polyedrum</i>	10 (25), 43 (107), 93 (234), 417 (1041), 819 (2048), 1629 (4072)	1–4

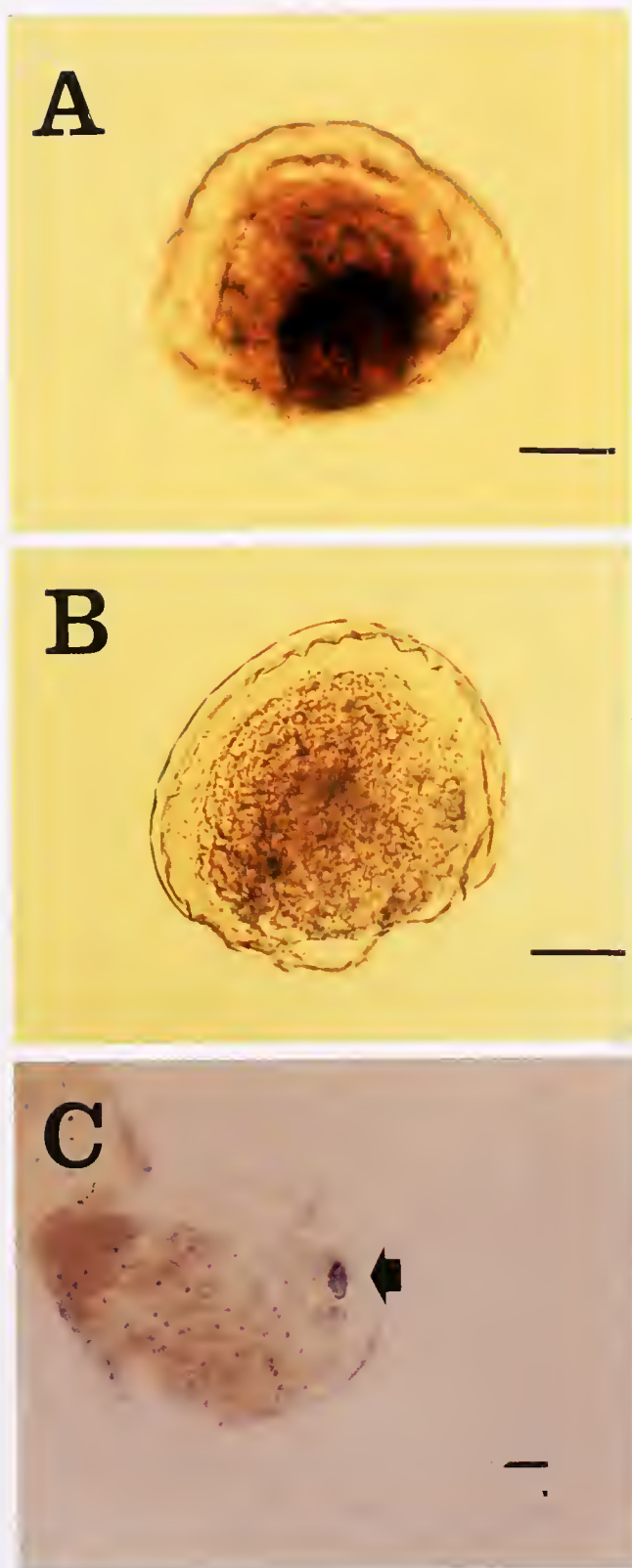


Figure 1. *Mytilus galloprovincialis* larva fed (A) and unfed (B) red-tide dinoflagellates. An undigested *Prorocentrum micans* cell (arrow) from the crushed stomach of a larva (C). Scale bars are 50 μm in (A & B) and 20 μm in (C).

illumination of $50 \mu\text{E m}^{-2} \text{s}^{-1}$ from cool white fluorescent light. One day later, prey inside the stomach of the larvae was almost digested and indiscernible by microscope examination. The abundance of *M. galloprovincialis* larvae was determined by enumerating larvae in three 1-mL Sedgwick-Rafter counting chambers (hereafter SRCs).

Initial densities of *M. galloprovincialis* larvae and target prey were established using an autopipette to deliver predetermined volumes of known densities to the bottles. Triplicate 270-mL PC experiment bottles (mixtures of predator and prey) and triplicate control bottles (prey only) were set up at each predator-prey combination. Triplicate control bottles containing only *M. galloprovincialis* larvae also were established. Thirty milliliters of f/2 medium were added to each bottle. Each bottle then was filled to capacity with freshly filtered seawater and capped. The bottles were placed on plankton wheels under the environmental conditions described above. To determine actual predator and prey densities at the beginning of the experiment, and after 24, 48, and 72 h incubation, 10-mL aliquots were removed from each bottle and fixed with 5% Lugol's solution, and all larvae and all or >200 prey cells in three 1-mL SRCs were enumerated. Prior to taking subsamples, the condition of *M. galloprovincialis* larvae and prey was assessed by looking through the surface of each capped bottle using a dissecting microscope. The bottles were filled again to capacity with freshly filtered seawater, capped, and placed on plankton wheels as described above. Dilution of the cultures associated with refilling the bottles was considered in calculating ingestion rates.

Ingestion and clearance rates of *M. galloprovincialis* larvae on RTDs were calculated using the equations of Frost (1972). The incubation time for calculating ingestion and clearance rates was 48 h.

To examine the occurrence of ingestion by *M. galloprovincialis* larvae on each prey species, 2-mL aliquots from each experimental and control bottle, preserved with Lugol's solution after 48 h incubation, were transferred into wells of 12-well plate chambers. Subsequently, 0.3 mL thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) of a 10^5 mg L^{-1} con-

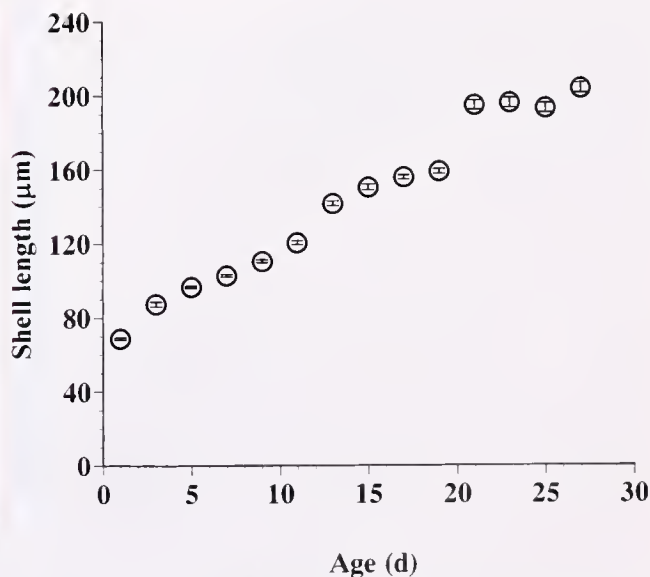


Figure 2. Shell length of *Mytilus galloprovincialis* larvae used for Experiments 1 to 7 as a function of larval age. Symbols represent treatment means ± 1 SE. $n = 37\text{--}403$ for each larval age.

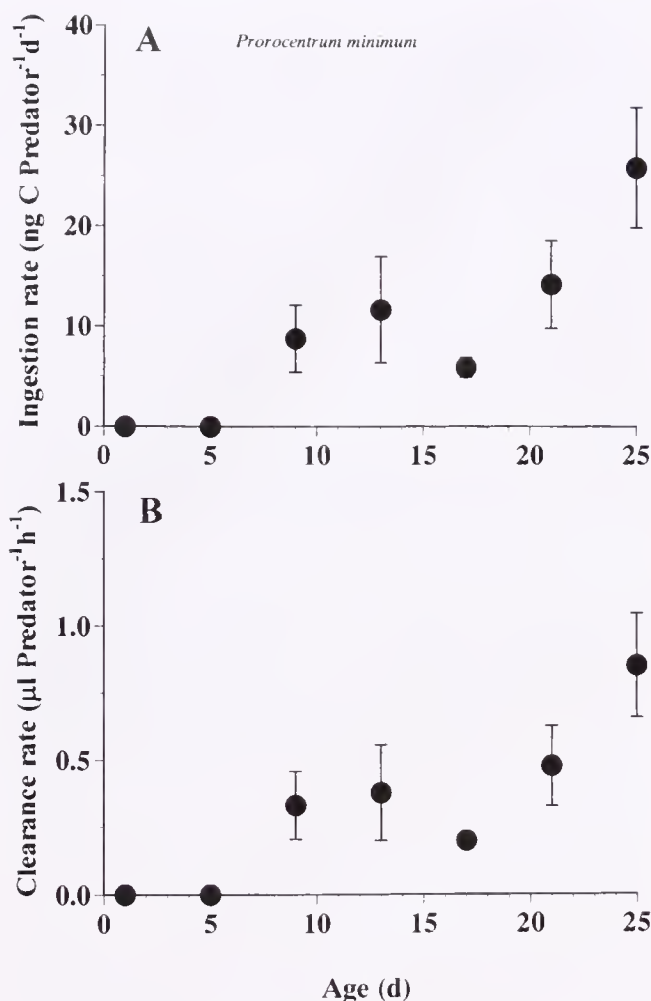


Figure 3. Ingestion (A) and clearance rates (B) of *Mytilus galloprovincialis* larvae on *Prorocentrum minimum* as a function of larval age. Symbols represent treatment means \pm 1 SE.

centration was added into each well to decolorize. One day later, the stomachs of the larvae were examined at 40–200 \times using Olympus compound and dissecting microscopes, and photographs were taken. Also, to find any undigested prey cells inside the stomachs of the larvae, approximately 20 *M. galloprovincialis* larvae fed on *Prorocentrum micans* were rinsed and carefully crushed using a very thin needle. We chose *P. micans* because it has an easily discernible shape. Photographs of the contents from the stomach were taken.

The shell lengths of *M. galloprovincialis* larvae at the beginning of each experiment and after 48 h incubation were measured using an image analyzing system; each individual larva was observed at a magnification of 20 \times , and its image was recorded using a Toshiba Model IK-642K CCD camera attached to a stereo-zoom microscope (Nikon, SMZ-U). Measurements of the shell length (the distance between the anterior and posterior ends of a shell) were conducted using the UTHSCSA Image Tool program. The shell lengths of 30 larvae at each age were measured.

Ingestion Rates as a Function of Prey Concentration

Experiments 8 to 13 were designed to measure ingestion and clearance rates of 25-day-old *M. galloprovincialis* larvae, as a

function of prey concentration, when feeding on unialgal diets of the red-tide dinoflagellates (Table 2).

The procedures for setting-up experiments, measuring predator and prey densities, and calculating ingestion and clearance rates were the same as described above, except that 80-mL PC bottles were used, 10 mL of f/2 medium were added into each bottle, and 5-mL aliquots were removed from each bottle and fixed with 5% Lugol's solution.

Ingestion rate data were fitted to a Michaelis–Menten equation:

$$IR = I_{\max}(x)/[K_{IR} + (x)] \quad (1)$$

where I_{\max} = the maximum ingestion rate (cells predator⁻¹ day⁻¹ or ng C predator⁻¹ day⁻¹); x = prey concentration (cells mL⁻¹ or ng C mL⁻¹), and K_{IR} = the prey concentration sustaining $\frac{1}{2} I_{\max}$.

RESULTS

Feeding and Shell Length of *Mytilus* Larvae

M. galloprovincialis larvae were able to ingest all red-tide dinoflagellates offered in the current study. The larvae fed on RTD in feeding currents produced by the ciliated velum. The stomach areas of *M. galloprovincialis* fed red-tide dinoflagellates were 20–

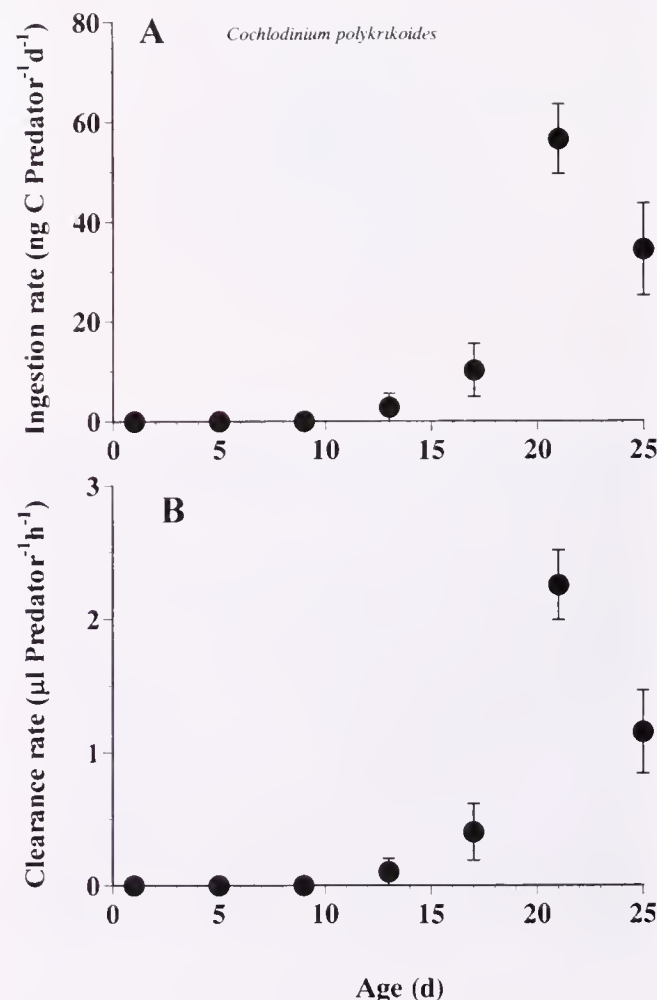


Figure 4. Ingestion (A) and clearance rates (B) of *Mytilus galloprovincialis* larvae on *Cochlodinium polykrikoides* as a function of larval age. Symbols represent treatment means \pm 1 SE.

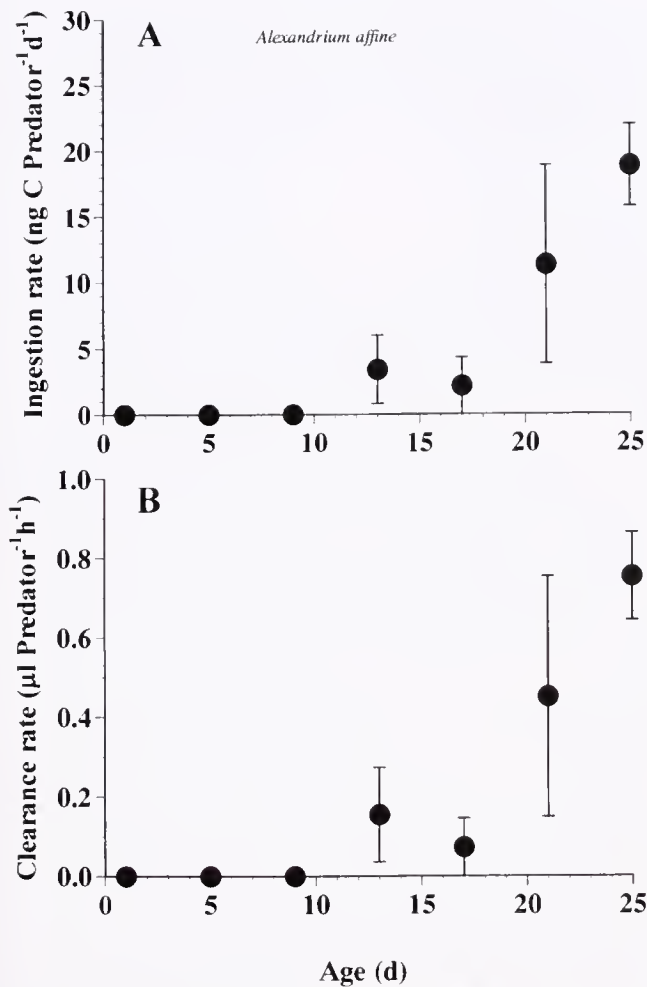


Figure 5. Ingestion (A) and clearance rates (B) of *Mytilus galloprovincialis* larvae on *Alexandrium affine* as a function of larval age. Symbols represent treatment means \pm 1 SE.

30% larger than those starved in the control bottles, and the color of the stomachs of fed larvae became dark brown, whereas that of unfed larvae was almost transparent (Figs. 1A and 1B). Undigested *Prorocentrum micans* cells from the crushed stomachs of the larvae also ascertained the ingestion of the prey species (Fig. 1C).

With increasing larval age (elapsed time after fertilization), the mean shell length of *M. galloprovincialis* larvae almost linearly increased from 69 to 204 μ m (Fig. 2).

Ingestion and Clearance Rates as a Function of Larval Age

The first feeding by *M. galloprovincialis* larvae on each red-tide dinoflagellate species occurred when the larvae were approximately 9–13 days old (Figs. 3–8); whereas that for *Isochrysis galbana* occurred at the larval age of 5 days (Fig. 9).

The ingestion rates of *M. galloprovincialis* larvae on *P. minimum* were undetectable when the larvae were 1–5 days old. However, they increased to 6–14 ng C predator⁻¹ day⁻¹ at the larval age of 9–21 days and reached 26 at 25 days (Fig. 3A). The clearance rates were 0.2–0.5 μ L predator⁻¹ h⁻¹ at the age of 9–21 days and reached 0.9 at 25 days (Fig. 3B).

The ingestion rates of *M. galloprovincialis* larvae on *Cochlodinium polykrikoides* were undetectable or very low when the larvae were 1–13 days old, but they rapidly increased to 34–56 ng C predator⁻¹ day⁻¹ at the larval age of 21–25 days (Fig. 4A). The clearance rates also were undetectable or very low at the larval age of 1–13 days, but increased to 1.3–2.3 μ L predator⁻¹ h⁻¹ at the larval age of 21–25 days (Fig. 4B).

The ingestion rates of *M. galloprovincialis* larvae on *Alexandrium affine* were undetectable or very low when the larvae were 1–17 days old, but they rapidly increased to 19 ng C predator⁻¹ day⁻¹ at the age of 25 days (Fig. 5A). The clearance rates also were undetectable or very low at the larval age of 1–17 days, but increased to 0.8 μ L predator⁻¹ h⁻¹ at the larval age of 25 days (Fig. 5B).

The ingestion rates of *M. galloprovincialis* larvae on *Scrippsiella trochoidea* were undetectable or very low when the larvae were 1–13 days old, but they increased rapidly to 19–20 ng C predator⁻¹ day⁻¹ at the age of 21–25 days (Fig. 6A). The clearance rates also were undetectable or very low at the larval age of 1–13 days, but increased to 0.3–0.7 μ L predator⁻¹ h⁻¹ at the larval age of 21–25 days (Fig. 6B).

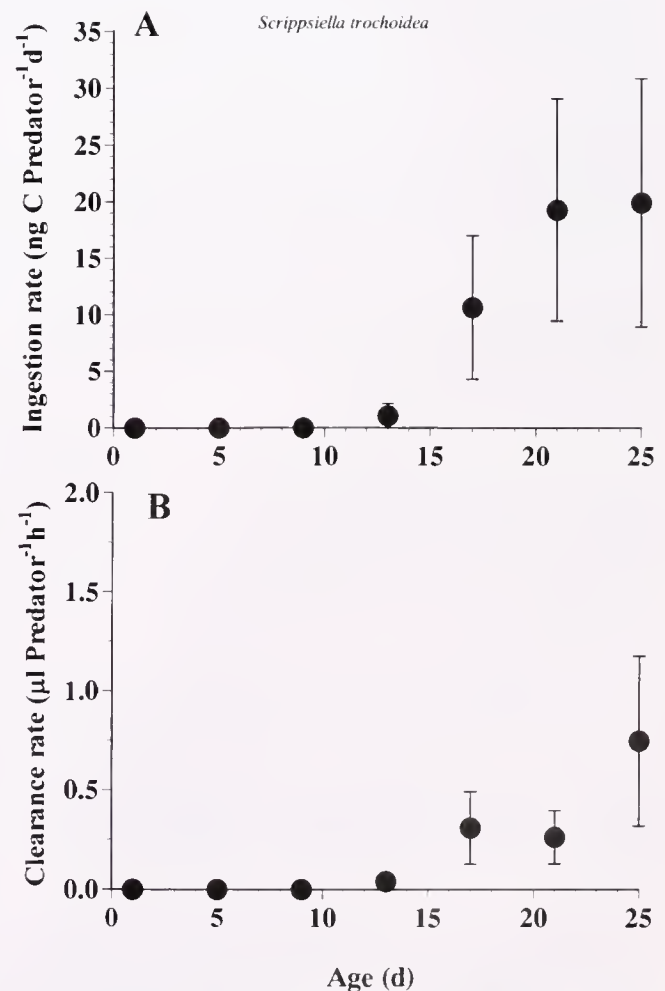


Figure 6. Ingestion (A) and clearance rates (B) of *Mytilus galloprovincialis* larvae on *Scrippsiella trochoidea* as a function of larval age. Symbols represent treatment means \pm 1 SE.

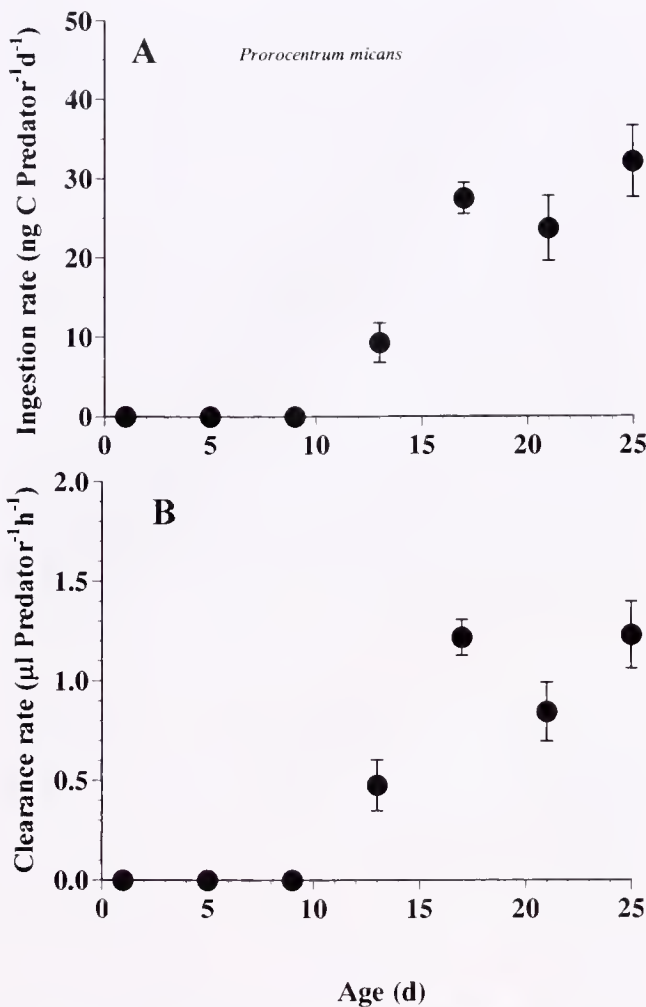


Figure 7. Ingestion (A) and clearance rates (B) of *Mytilus galloprovincialis* larvae on *Prorocentrum micans* as a function of larval age. Symbols represent treatment means \pm 1 SE.

The ingestion rates of *M. galloprovincialis* larvae on *Prorocentrum micans* were undetectable when the larvae were 1–9 days old, but they increased rapidly to 27–32 ng C predator⁻¹ day⁻¹ at the age of 17–25 days (Fig. 7A). The clearance rates were also undetectable or very low at the larval age of 1–9 days, but increased to 0.8–1.2 μ L predator⁻¹ h⁻¹ at the larval age of 17–25 days (Fig. 7B).

The ingestion rates of *M. galloprovincialis* larvae on *Lingulodinium polyedrum* were undetectable when the larvae were 1–9 days old, but they were 10–11 ng C predator⁻¹ day⁻¹ at the age of 13–21 days (Fig. 8A). The rates reached a maximum of 27 ng C predator⁻¹ day⁻¹ at the age of 25 days. The clearance rates were also undetectable when the larvae were 1–9 days old, but they were 0.1–0.4 μ L predator⁻¹ h⁻¹ at the age of 13–21 days (Fig. 8B). The rate reached a maximum of 1.1 μ L predator⁻¹ h⁻¹ at the age of 25 days.

The ingestion rates of *M. galloprovincialis* larvae on *Isochrysis galbana* were undetectable when the larvae were 1 day old, but they were 19–32 ng C predator⁻¹ day⁻¹ at the age of 5–17 days (Fig. 9A). The rates reached maximum of 111 ng C predator⁻¹ day⁻¹ at the age of 21 days. The clearance rates were also undetectable when the larvae were 1 day old, but they were 1.0–1.7 μ L preda-

tor⁻¹ h⁻¹ at the age of 5–17 days (Fig. 9B). The rate reached a maximum of 7.8 μ L predator⁻¹ h⁻¹ at the age of 25 days.

No dead *M. galloprovincialis* larvae were found upon examination with a dissecting microscope prior to taking subsamples in these experiments.

Ingestion and Clearance Rates as a Function of Prey Concentration

In Experiments 8 to 13, the ingestion rates of 25-day-old *M. galloprovincialis* larvae on unialgal diets of *P. minimum*, *C. polykrikoides*, *A. affine*, *S. trochoidea*, *P. micans*, and *L. polyedrum* increased rapidly with increasing prey concentration up to 1000–2200 ng C mL⁻¹, but were almost saturated at higher prey concentration (Figs. 10–15). When the data were fitted to Eq. 1, the maximum ingestion rates of *M. galloprovincialis* larvae in ng C predator⁻¹ day⁻¹ and (prey cells predator⁻¹ day⁻¹) were 69 (99) for *C. polykrikoides*, 56 (60) for *P. micans*, 45 (18) for *L. polyedrum*, 26 (173) for *P. minimum*, 21 (25) for *S. trochoidea*, and 14 (18) for *A. affine* (Table 3). Maximum clearance rates of *M. galloprovincialis* larvae were 11.4 μ L predator⁻¹ h⁻¹ for *L. polyedrum*, 8.4 for *P. micans*, 3.8 for *A. affine*, 3.5 for *S. trochoidea*, 2.8 for *C. polykrikoides*, and 1.5 for *P. minimum*.

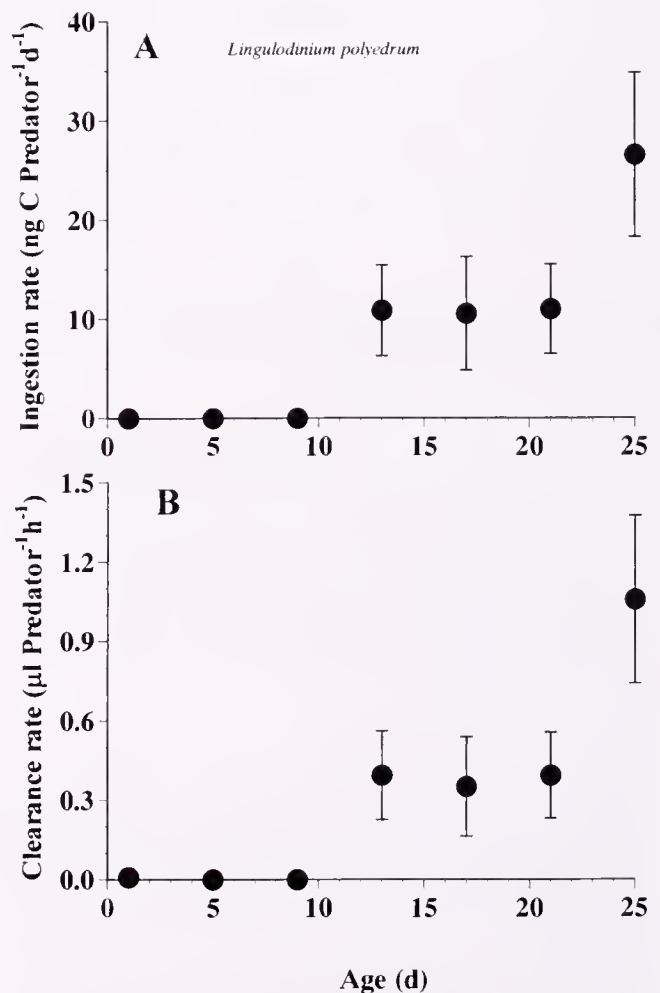


Figure 8. Ingestion (A) and clearance rates (B) of *Mytilus galloprovincialis* larvae on *Lingulodinium polyedrum* as a function of larval age. Symbols represent treatment means \pm 1 SE.

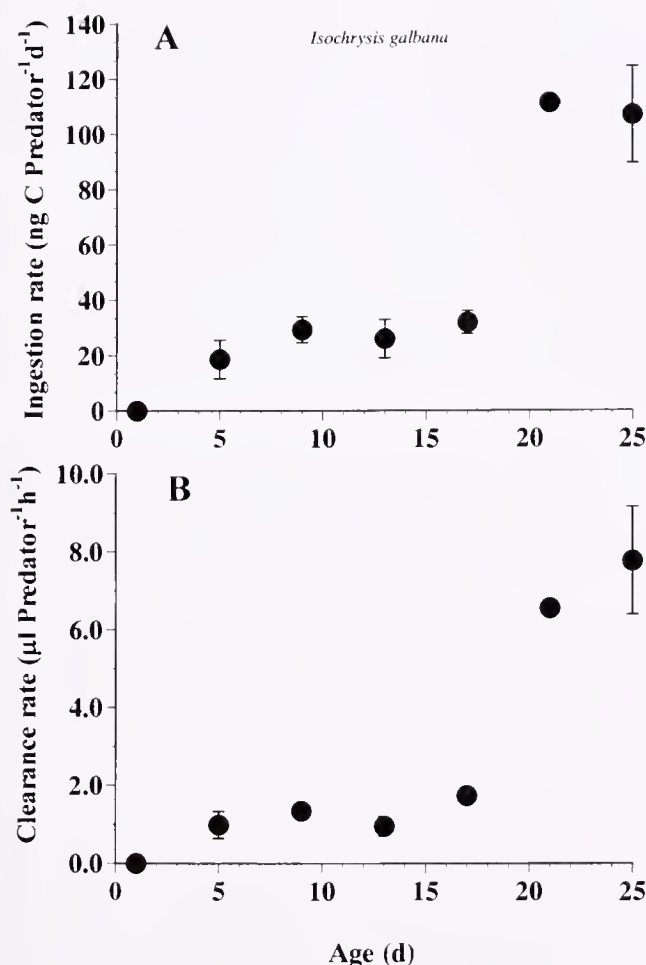


Figure 9. Ingestion (A) and clearance rates (B) of *Mytilus galloprovincialis* larvae on *Isochrysis galbana* as a function of larval age. Symbols represent treatment means \pm 1 SE.

DISCUSSION

Prey Species and Feeding Rates as a Function of Larval Age

There has been no report on the feeding by larvae in the genus *Mytilus* on red-tide dinoflagellates. *M. galloprovincialis* larvae were able to feed on all red-tide dinoflagellate prey offered in the current study. Thus, *M. galloprovincialis* larvae have diverse prey species. In the phylum mollusca, larvae of the oyster *Crassostrea virginica* have been shown to feed on the dinoflagellate *P. minimum* (Wikfors & Smolowitz, 1995), and larvae of the gastropod *Philine aperta* feed on the dinoflagellates *Heterocapsa triquetra* and *Scrippsiella feroense* (Hansen 1991). Further studies on feeding by other molluscan larvae on diverse red-tide dinoflagellates are necessary to better understand their interactions.

Mytilus edulis larvae have been known to ingest particles of 1–9 μ m (Riisgård et al. 1980, Sprung 1984b). However, *M. galloprovincialis* larvae are able to ingest red-tide dinoflagellates whose ESDs are 12–38 μ m (Table 1). The change in color of the stomachs of the larvae and undigested prey cells found in the crushed stomachs indicated their ingestion (Fig. 1). The mouths of the larvae may be very flexible for ingesting large prey cells or different species in the genus *Mytilus* may have different prey size limitations.

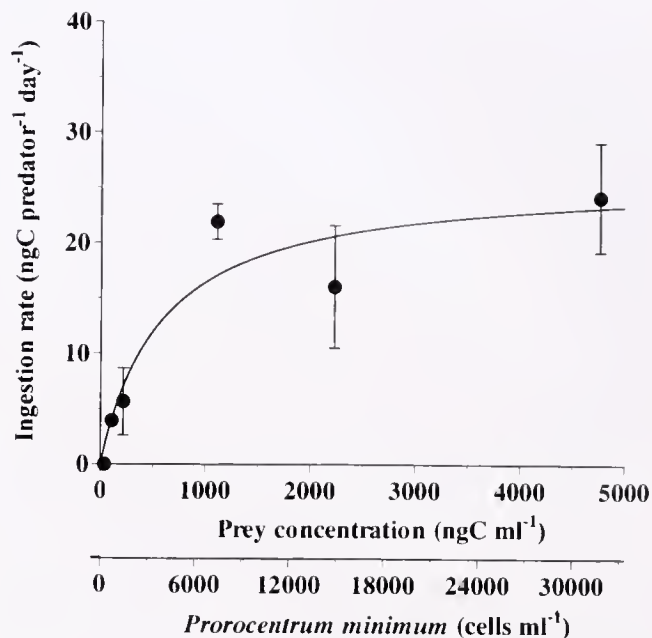


Figure 10. Ingestion rates of 25-day-old *Mytilus galloprovincialis* larvae on *Prorocentrum minimum* as a function of mean prey concentration. Symbols represent treatment means \pm 1 SE. The curves are fitted by a Michaelis-Menten equation (Eq. 1) using all treatments (see Table 3).

The first feeding by *M. galloprovincialis* larvae on each red-tide dinoflagellate species occurred when the larvae were approximately 9–13 days old (Figs. 3–8), whereas that for *I. galbana* occurred at the larval age of 5 days (Fig. 9). Much larger size of red-tide dinoflagellates might delay the larval first feeding.

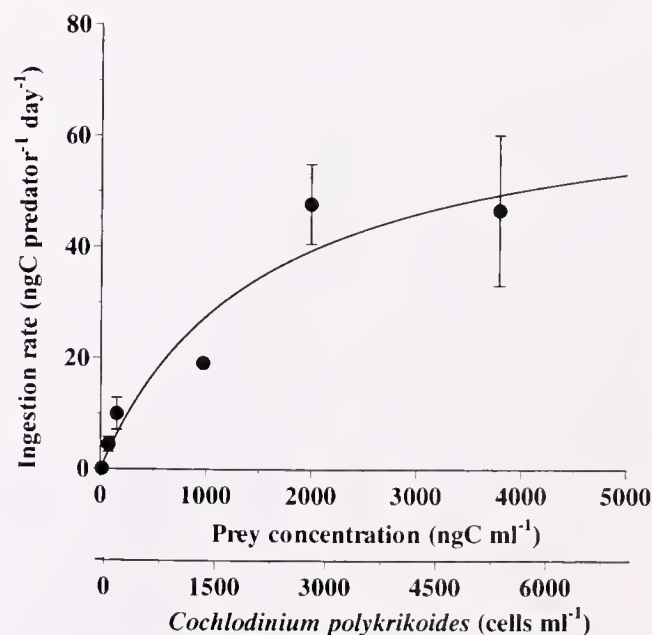


Figure 11. Ingestion rates of 25-day-old *Mytilus galloprovincialis* larvae on *Cochlodinium polykrikoides* as a function of mean prey concentration. Symbols represent treatment means \pm 1 SE. The curves are fitted as in Fig. 10.

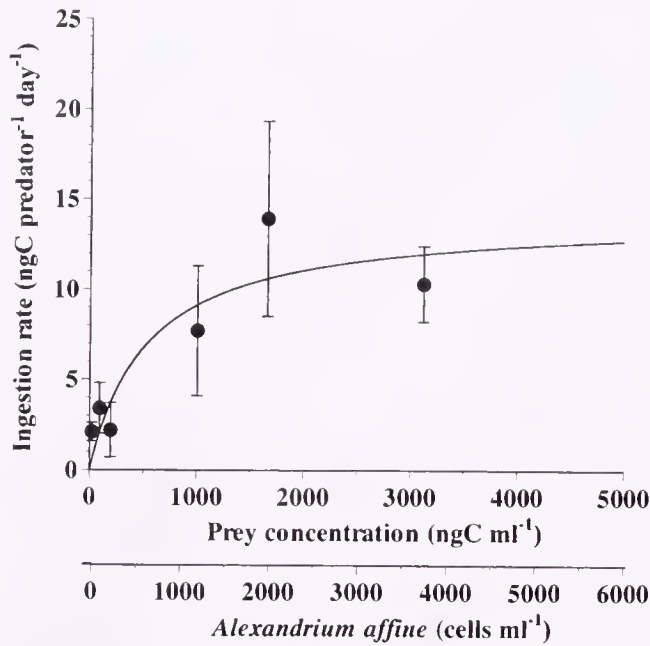


Figure 12. Ingestion rates of 25-day-old *Mytilus galloprovincialis* larvae on *Alexandrium affine* as a function of mean prey concentration. Symbols represent treatment means \pm 1 SE. The curves are fitted as in Fig. 10.

The ingestion and clearance rates of *M. galloprovincialis* larvae on red-tide dinoflagellates at the larval ages of 21–25 days ($14\text{--}56\text{ ng C predator}^{-1}\text{ day}^{-1}$ and $0.5\text{--}2.3\text{ }\mu\text{L predator}^{-1}\text{ h}^{-1}$, respectively) measured in Experiments 1 to 7 were lower than those on *I. galbana* ($107\text{--}111\text{ ng C predator}^{-1}\text{ day}^{-1}$ and $6.5\text{--}7.8\text{ }\mu\text{L predator}^{-1}\text{ h}^{-1}$) (Figs. 3–9). Therefore, red-tide dinoflagellates are less preferred prey for *M. galloprovincialis* larvae than *I. galbana*.

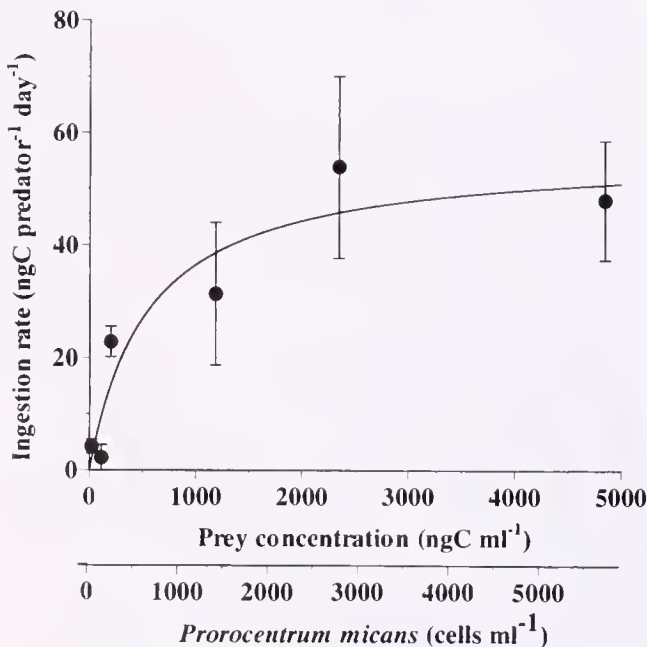


Figure 13. Ingestion rates of 25-day-old *Mytilus galloprovincialis* larvae on *Scrippsiella trochoidea* as a function of mean prey concentration. Symbols represent treatment means \pm 1 SE. The curves are fitted as in Fig. 10.

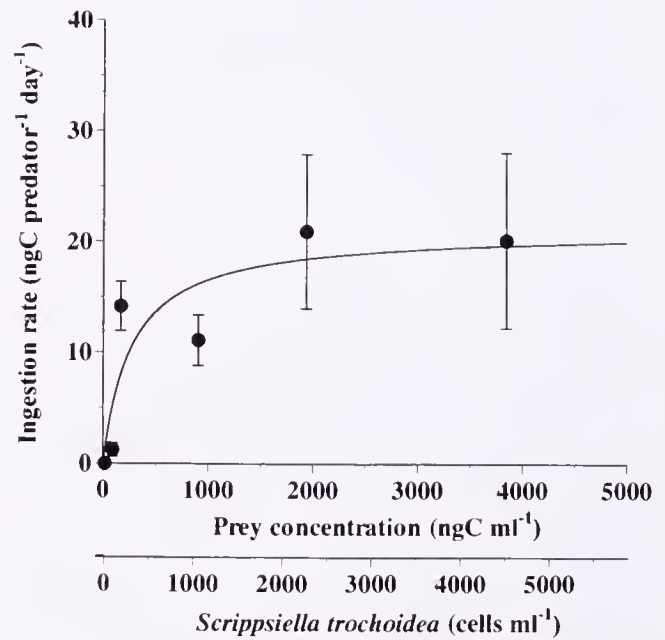


Figure 14. Ingestion rates of 25-day-old *Mytilus galloprovincialis* larvae on *Prorocentrum micans* as a function of mean prey concentration. Symbols represent treatment means \pm 1 SE. The curves are fitted as in Fig. 10.

Ingestion and Clearance

Data from this study show that the maximum ingestion rates of 25-day-old *M. galloprovincialis* larvae on each red-tide dinoflagellate species measured in Experiments 8 to 13 are poorly correlated with prey cell volume (Fig. 16). This relationship suggests that prey cell volume does not have an affect on ingestion by the larvae

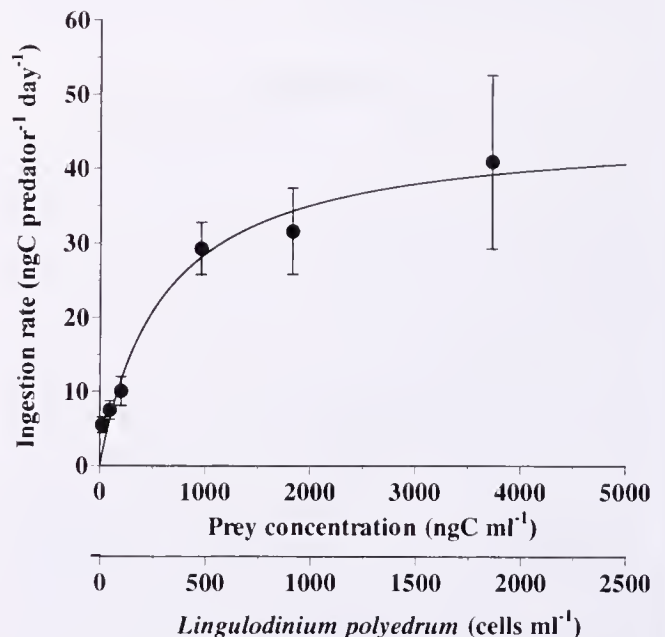


Figure 15. Ingestion rates of 25-day-old *Mytilus galloprovincialis* larvae on *Lingulodinium polyedrum* as a function of mean prey concentration. Symbols represent treatment means \pm 1 SE. The curves are fitted as in Fig. 10.

TABLE 3.

Grazing data for *Mytilus galloprovincialis* larvae.*

Figures	Species	I_{\max}	K_{IR}	r^2
10	<i>Prorocentrum minimum</i>	26	577	0.72
11	<i>Cochlodinium polykrikoides</i>	69	1510	0.73
12	<i>Alexandrium affine</i>	14	539	0.48
13	<i>Scrippsiella trochoidea</i>	21	269	0.50
14	<i>Prorocentrum micans</i>	56	538	0.61
15	<i>Lingulodinium polyedrum</i>	45	590	0.73

* Parameters are for functional response from Eq. 1 as presented in Figs. 10–15. I_{\max} (maximum ingestion rate, ng C predator⁻¹ day⁻¹), K_{IR} (prey concentration sustaining 0.5 I_{\max} , ng C mL⁻¹).

of red-tide dinoflagellates. Thus, factors other than prey cell volume may be important to the feeding activity of the larvae. Maximum ingestion rates of the larvae on *C. polykrikoides* and *P. micans* were much higher than those for *S. trochoidea* and *A. affine*, even though these prey species are similar in cell volume. However, these results are difficult to interpret. The C:N ratios of *C. polykrikoides* (7.7) and *P. micans* (8.7) are similar to or higher than those for *S. trochoidea* (5.6) and *A. affine* (8.4) (Jeong et al., unpublished data). Thus, nutritional values of prey species may not be responsible for these different maximum ingestion rates. Trochophore larvae of the oyster *Crassostrea gigas* 17 h after fertilization have been shown to be killed when exposed to toxic dinoflagellates (Matsuyama et al. 2001). However, *S. trochoidea* and *A. affine* are nontoxic dinoflagellates (our data). Regarding cell shape, *C. polykrikoides* and *P. micans* cells are compressed, whereas *S. trochoidea* and *A. affine* are spherical. Therefore, the compressed cells may be easier for the larvae to ingest than the spherical cells. However, to determine the exact cause of this pattern, further study is necessary.

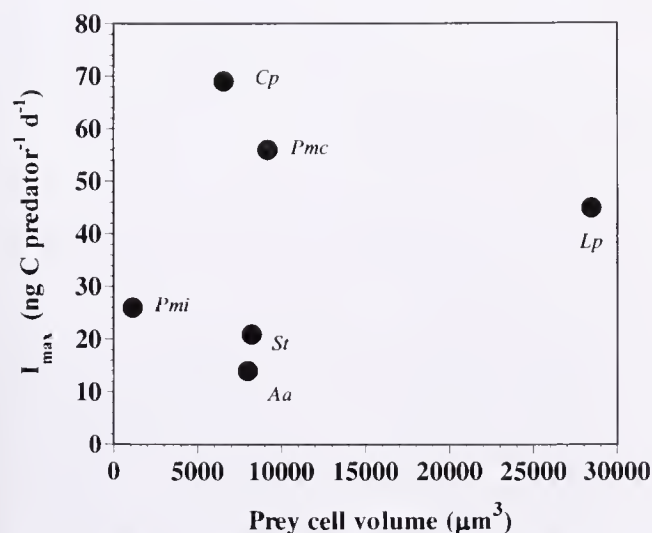


Figure 16. Maximum ingestion rates (I_{\max}) of 25-day-old *Mytilus galloprovincialis* larvae on 6 red-tide dinoflagellate species as a function of prey cell volume. Aa: *Alexandrium affine*, Cp: *Cochlodinium polykrikoides*, Lp: *Lingulodinium polyedrum*, Pmc: *Prorocentrum micans*, Pmi: *Prorocentrum minimum*, and St: *Scrippsiella trochoidea*.

The maximum ingestion (I_{\max}) and clearance rates (C_{\max}) of 25-day-old *M. galloprovincialis* larvae on red-tide dinoflagellates obtained in this study (69 ng C predator⁻¹ day⁻¹ and 11.4 μL predator⁻¹ h⁻¹, respectively) are comparable to or lower than those for *M. edulis* larvae on microflagellates (37–160 ng C predator⁻¹ day⁻¹ and 11–88 μL predator⁻¹ h⁻¹, respectively) (Bayne 1965, Riisgard et al. 1980, Riisgard et al. 1981, Jespersen & Olsen 1982, Sprung 1984b), when corrected to 15°C using $Q_{10} = 3.4$ (Hansen et al. 1997). The smaller size of *M. galloprovincialis* larvae (193 μm) with respect to *M. edulis* larvae (250) might be responsible for the former predator's lower C_{\max} .

The I_{\max} and C_{\max} of 25-day-old *M. galloprovincialis* larvae on each red-tide dinoflagellate species are higher than those previously reported for a mixotrophic dinoflagellate, heterotrophic dinoflagellates, or a small ciliate, but much lower than those for large ciliates on the same prey (Table 4). For example, the I_{\max} of *M. galloprovincialis* larvae on *Lingulodinium polyedrum* obtained in this study (45 ng C predator⁻¹ day⁻¹) is higher than those of *Polykrikos kofoidii* (16 ng C predator⁻¹ day⁻¹), *Tiarina fusus* (15), *Protoperdinium cf. divergens* (8), *Fragilidium cf. mexicanum* (5), and *Protoperdinium crassipes* (3), but much lower than that of *Strombidinopsis* sp. (147 ng C predator⁻¹ day⁻¹), when corrected to 15°C using $Q_{10} = 2.8$ (Hansen et al. 1997). The C_{\max} of *M. galloprovincialis* larvae on *L. polyedrum* (11.4 μL predator⁻¹ h⁻¹) is also higher than those of *P. kofoidii* (3.9), *T. fusus* (3.0), *F. cf. mexicanum* (2.6), *P. cf. divergens* (0.5), and *P. crassipes* (0.3), but much lower than that of *Strombidinopsis* sp. (73). This pattern is maintained in *S. trochoidea*, *C. polykrikoides*, *P. minimum*, and *P. micans* prey. This evidence suggests that engulfing prey in the feeding current produced by the ciliated velum near the mouth (*Mytilus* larvae) is a more effective feeding mechanism than engulfing prey captured by a tow filament (*Polykrikos* spp.) or palium feeding on prey captured by a tow filament (*Protoperdinium* spp.), but less effective than engulfing prey using rows of cilia in the mouth (*Strombidinopsis* spp. and *Favella* spp.).

Ecological Importance

In the current study, *M. galloprovincialis* larvae fed on red-tide dinoflagellates without mortality after 72 h exposure to considerable high prey concentrations. Thus, the larvae are able to survive during and/or after red tides dominated by these dinoflagellates. Also, dinoflagellates are one of the most abundant phytoplankters in coastal waters, and thus the bivalve larvae may develop healthily by feeding on commonly distributed dinoflagellates. *M. galloprovincialis* larvae, one component of microzooplankters, exhibited higher maximum ingestion and clearance rates than previously reported for other microzooplankters such as the *Fragilidium cf. mexicanum* (mixotrophic dinoflagellate), the *Protoperdinium cf. divergens*, *Polykrikos kofoidii* (heterotrophic dinoflagellates), or *Tiarina fusus* (small ciliate), but lower rates than *Strombidinopsis* spp. and *Favella* spp. (large ciliates) when fed the same prey species. Thus, *Mytilus* larvae may compete with some microzooplankters for dinoflagellate prey.

ACKNOWLEDGMENTS

The authors thank Kwang Young Kim, Jae Seong Kim, Yeong Du Yoo, and Kyeong A Seong for technical support. This paper was funded by grants from MOST & KOSEF (R12-1999-027-12000-0) and from MOST & KISTEP (M1-0302-00-0068).

TABLE 4.

Comparison of ingestion and clearance rates of *Mytilus galloprovincialis* larvae and protistan predators on the same red-tide algal prey.*

Prey Species	Predator	PV	I_{\max}	C_{\max}	Reference
<i>Lingulodinium polyedrum</i>	<i>Mytilus galloprovincialis</i> larvae (ML)	4240	45	11.4	This study
	<i>Tiarina fusu</i> (NC)	23	15	3.0	Jeong et al. (2002)
	<i>Polykrikos kofoidii</i> (HD)	43	16	3.9	Jeong et al. (2001)
	<i>Proto-peridinium</i> cf. <i>divergens</i> (HD)	119	8	0.5	Jeong and Latz (1994)
	<i>Proto-peridinium crassipes</i> (HD)	204	3	0.3	Jeong and Latz (1994)
	<i>Fragilidium</i> cf. <i>mexicanum</i> (MD)	85	5	2.6	Jeong et al. (1999a)
<i>Scrippsiella trochoidea</i>	<i>Strombidinopsis</i> spp. (NC)	560	147	72.9	Jeong et al. (1999b)
	<i>Mytilus galloprovincialis</i> larvae (ML)	4240	21	3.5	This study
	<i>Tiarina fusu</i> (NC)	23	7	0.1	Jeong et al. (2002)
	<i>Polykrikos kofoidii</i> (HD)	43	11	0.7	Jeong et al. (2001)
	<i>Strombidinopsis</i> spp. (NC)	560	137	27.2	Jeong et al. (1999b)
	<i>Favella</i> spp. (TC)		157	28.5	Stoecker et al. (1981)
<i>Cochlodinium polykrikoides</i>	<i>Mytilus galloprovincialis</i> larvae (ML)	4240	69	2.8	This study
	<i>Strombidinopsis</i> spp. (NC)	560	234	33.0	Jeong et al. (1999b)
<i>Prorocentrum minimum</i>	<i>Mytilus galloprovincialis</i> larvae (ML)	4240	26	7.2	This study
	<i>Strombidinopsis</i> spp. (NC)	560	177	73.0	Jeong et al. (1999b)
<i>Prorocentrum micans</i>	<i>Mytilus galloprovincialis</i> larvae (ML)	4240	26	7.2	This study
	<i>Polykrikos kofoidii</i> (HD)	43	3	1.5	Jeong et al. (2001)

* Rates are corrected to 15°C using $Q_{10} = 2.8$ (Hansen et al. 1997). PV (Predators' volume as $\times 10^3 \mu\text{m}^3$); I_{\max} (maximum ingestion rate in ng C predator⁻¹ day⁻¹); C_{\max} (maximum clearance rate as $\mu\text{L predator}^{-1} \text{h}^{-1}$); NC (naked ciliate); TC (tintinnid ciliate); HD (heterotrophic dinoflagellate); MD (mixotrophic dinoflagellate); ML (metazoan larvae).

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COMPARATIVE GROWTH PERFORMANCE OF EARLY JUVENILE *HALIOTIS ASININA* FED VARIOUS ARTIFICIAL DIETS

M. KRUATRACHUE,^{1,*} S. SAWATPEERA,² Y. CHITRAMVONG,¹ P. SONCHAENG,²
E. S. UPATHAM,³ AND S. SANGPRADUB⁴

¹Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400 Thailand; ²Institute of Marine Science, Burapha University, Chonburi 20131, Thailand; ³Faculty of Science, Burapha University, Chonburi 20131, Thailand; ⁴Coastal Aquaculture Development Center, Prachuap Khiri Khan 77000, Thailand

ABSTRACT The purpose of this study was to compare artificial diets for early juvenile abalone, *Haliotis asinina*, containing different sources of protein. Juvenile *H. asinina* with mean initial shell length of 5.3–5.6 mm were fed artificial diets for 90 days. Diets contained 30% crude protein from different sources: casein, fish meal, soybean meal, and rice bran. The diets were fed to abalone at 5–10% body weight once daily in the afternoon. Abalone fed *Acanthophora* sp. served as a control. The results showed that the abalone fed fresh *Acanthophora* sp. and those receiving the casein-based diet had the highest growth rates in shell length (96.7 ± 8.0 $\mu\text{m/day}$ and 96.3 ± 6.7 $\mu\text{m/day}$, respectively). Those fed the casein-based diet showed the highest growth rate in weight (8.6 ± 0.3 mg/day). The best survival rates were found in abalone fed *Acanthophora* sp., the soybean-based diet and the casein-based diet (88.9%, 81.1%, and 78.9%, respectively). The casein diet yielded the maximum rate of growth and survival.

KEY WORDS: comparative growth performance, early juvenile, artificial diets, *Haliotis asinina*

INTRODUCTION

The donkey's ear abalone, *Haliotis asinina* Linnaeus, a very promising commercial abalone in the Southeast Asia region, is a tropical species. It is distributed widely in coastal reef zones of Southeast Asia. In Thailand, research into abalone culturing techniques began in the early 1990s (Singhagruiwan 1989, Singhagruiwan & Doi 1993, Jarayabhand & Paphavasit 1996). Feeding, growth, and survival (Singhagruiwan 1991a, 1991b, Singhagruiwan & Sasaki 1991, Singhagruiwan 1993, Kunavongdate et al. 1995, Upatham et al. 1998, Kruatrachue et al. 2000) have been investigated for adult abalone, but little work has been done on juvenile *H. asinina*.

Culture of *H. asinina* in Thailand is divided into two phases. The first phase is the culture of newly settled spats on corrugated plastic plates coated with diatoms until a shell length of 5–10 mm is attained. The second phase is the culture of juveniles on corrugated cement plates with seaweed until they reach a marketable size (40–50 mm in shell length) (Singhagruiwan & Doi 1993). There are many problems, however, in using natural seaweed, such as seasonality and accessibility (Uki & Watanabe 1992). Artificial diets could solve these problems.

In recent years, there has been a rapid increase in the number of research groups developing artificial diets to supplement or replace seaweeds in abalone culture (Uki & Watanabe 1992, Viana et al. 1993, Fleming et al. 1996, Britz 1996a, 1996b, Capinpin & Corre 1996, Moss 1997, Corazani & Illanes 1998, Lopez et al. 1998, Chen & Lee 1999, Kruatrachue et al. 2000, Serviere-Zaragoza et al. 2001, Boarder & Shpigel 2001, Shipton & Britz 2001, Jackson et al. 2001). Growth of abalone feeding on macroalgae and artificial diets has been studied for *H. discus* Reeve (Ogino & Ohta 1963), *H. discus hannai* Ino (Uki et al. 1985, Nie et al. 1986, Uki et al. 1986a, 1986b, Corazani & Illanes 1998), *H. fulgens* Philippi (Viana et al. 1993, Serviere-Zaragoza et al. 2001), *H. asinina* (Capinpin & Corre, 1996, Upatham et al. 1998, Capinpin et al. 1999, Bautista-Teruel & Millamena 1999, Kruatrachue

et al. 2000, Jackson et al. 2001), *H. iris* Gmelin (Stuart & Brown 1994), *H. tuberculata* Linnaeus (Koike et al. 1979, Mgaya & Mercer 1995, Lopez et al. 1998), *H. laevigata* Donovan (Morrison & Whittington 1991), *H. rufescens* Swainson (Corazani & Illanes 1998), *H. australis* Gmelin (Moss 1997), *H. diversicolor super-texta* Lischke (Chen & Lee 1999), and *H. midae* Linnaeus (Britz 1996a, Knauer et al. 1996, Shipton & Britz 2001). Among these extensive studies, only a few reported on early juvenile growth of abalone.

For commercial abalone culture, it is important to enhance the growth at all stages and therefore information is required on the dietary requirements of each size class. This study was carried out to investigate the growth and survival of early juvenile *H. asinina* fed four artificial diets with different sources of protein: casein, fish meal, soybean meal, and rice bran.

MATERIALS AND METHODS

Abalone

Three-month-old juvenile *H. asinina* (5.5 ± 0.2 mm in length, 45.3 ± 3.7 in weight) were obtained from the Coastal Aquaculture Development Center, Prachuap Khiri Khan Province, Thailand. They were placed in the rearing system for acclimatization and fed *Acanthophora* sp. seaweed for two weeks before collection of initial data.

Preparation of Diets

Diet formulations are presented in Table 1. The diets were prepared following Uki et al. (1986a), using different sources of protein-casein, fishmeal, soybean meal, and rice bran. The vitamin mix in Cetavit 1,000 g was obtained from the Eastern Marine Co., Ltd., Thailand, and mineral mix in Premix 1,000 g was obtained from Chanaphant Industry Co., Ltd., Thailand (Table 2). Equivalent amounts of vitamins and minerals were added to all diets (Table 1).

All dry ingredients were mixed together and then cod liver oil and vegetable oil added. The sodium alginate was heated in 60–80 mL distilled water at 60–80 C, until it melted, then poured into the

*Corresponding author. E-mail: scmkt@mahidol.ac.th

TABLE 1.

Composition of four artificial diets for juvenile abalone *H. asinina* (% inclusion).

Ingredients	Protein Based Diet (%)			
	Casein	Fish Meal	Soybean Meal	Rice Bran
Casein	25			
Fish meal		39		
Soybean meal			68	
Rice bran				68
Sodium alginate	16	16	16	16
Dextrin	21.5	14.5	0	0
Cellulose	21.5	14.5	0	0
Vitamins	5	5	5	5
Minerals	5	5	5	5
Cod liver oil	2.5	2.5	2.5	2.5
Vegetable oil	2.5	2.5	2.5	2.5
Calcium chloride	1	1	1	1

mixed ingredients and kneaded immediately to obtain a homogeneous paste. The paste was flattened with a kitchen roller to a thickness of 0.5 cm. Pieces 1.5 × 1.5 cm in dimension were cut and stored in the freezer until feeding.

Experimental Procedure

There were four dietary treatments: (1) casein-based diet; (2) fish meal-based diet; (3) soybean meal-based diet; and (4) rice bran-based diet. Fresh seaweed (*Acanthophora* sp.) was fed to the control group. Early juvenile abalone (5.3–5.6 mm in shell length, 40–50 mg in weight) were kept in rectangular 5-L plastic aquaria. Thirty abalone were allocated to each trial aquarium (three replicates per diet), and all 12 aquaria were floated in 700-L fiberglass tanks under a close system with aeration and filter system. The tanks were covered to prevent animals from escaping. UV-sterilized and filtered seawater (salinity 32–34 ppt, pH 8.4–8.5) was changed every morning for 90 days. The abalone were fed every afternoon with artificial diets and macroalgae at 5–10% of their body weights. Any uneaten food was collected the following morning to estimate the feed consumed and the food conversion rate (FCR). The light/dark sequence was 12h/12h and the temperature was 29–32 °C. Water quality parameters (DO, pH, and temperature) were randomly measured weekly. The total ammonia and the total nitrite were within the standard ranges (0.013–0.191 ppm and 0.016–0.053 ppm, respectively). The dry weight of artificial diets and algae were determined by drying in a hot air oven at 80 °C until a constant weight was reached. Growth was measured bi-weekly as gain in weight and shell length and expressed in terms of shell length (μm/day) and body weight (mg/day). Mortality was also recorded every two weeks.

The FCR was calculated on the basis of the total dry weight gain of abalone and the dry weight of feed consumed as follows (Leighton & Boolootian 1963):

$$\text{FCR} = \frac{\text{Dry weight feed consumed (g)}}{\text{Dry weight gain (g)}}$$

At the beginning of the experiment, 15 animals were sampled out to determine their total dry weight. Similarly, at the end of the experiment (90 days), the total dry weight of those animals that

TABLE 2.

Composition of vitamins and minerals mixes.

Composition	Weight
Vitamins	
Vitamin A	15,000,000 units
Vitamin D ₃	3,000,000 units
Vitamin C	83.0 g
Vitamin E	27.5 g
Vitamin K	4.67 g
Vitamin B ₁	25.0 g
Vitamin B ₂	25.0 g
Vitamin B ₆	5.0 g
Vitamin B ₁₂	0.05 g
Nicotinamide	20.0 g
Calcium-D-Panthenate	5.0 g
Folic acid	0.4 g
Minerals	
Manganese	5,400 g
Iron	14,200 g
Copper	1,000 g
Zinc	2,900 g
Sodium	3,300 g
Iodine	0.019 mg
Potassium	0.9 g
Cobalt	1.1 g
Medium	971.18 g

survived from each treatment was determined. These animals were weighed and dried at 80 °C until a constant weight was reached.

Statistical Analysis

Differences in growth and survival rates, and FCR among the different dietary treatments were determined by one-way ANOVA. The multiple comparisons, Duncan's multiple range test, was further used to determine significant differences between treatments. SPSS for Windows (Version 6.0) was the statistical software used for all statistical analysis. $P < 0.05$ was used as the significance level.

RESULTS

Growth Rate

The results obtained from each artificial diet and the control diet are shown in Table 3 and Figures 1 and 2. Growth rates (shell length and body weight increase/day) of *H. asinina* were significantly different ($P < 0.05$) among feed treatments. In terms of shell length, three statistically different groups could be recognized ($P < 0.05$): (1) control and casein-based diet; (2) fish meal-based and soybean meal-based diets; and (3) rice bran-based diet (Table 3). Four statistically different groups ($P < 0.05$) could be differentiated on weight increase: (1) casein-based diet; (2) control; (3) fish meal-based and soybean meal-based diets; and (4) rice bran-based diet (Table 3).

Shell Length

The maximum cumulative increases in shell length were 161.2% and 164.7% for the control and casein-based diet, respectively (Fig. 1). These equated to growth rates of 96.7 and 96.3 μm/day, respectively (Table 3). Lower cumulative increases of shell length were obtained with abalone fed fish meal-based (111.2%) and soybean meal-based diets (107.8%) (Fig. 1). These

TABLE 3.

Growth and survival rates of *H. asinina* fed different artificial diets over a period of 90 days.

Diet	Survival Rate (%)	Shell Length (mm)		Body Wet Weight (mg)		% Cumulative Increase		Growth	
		Initial	Final	Initial	Final	Length	Weight	Length ($\mu\text{m}/\text{day}$)	Weight (mg/day)
Control	88.9 \pm 3.8 ^a	5.4 \pm 0.2	14.1 \pm 0.8 ^a	44.7 \pm 4.2	615.0 \pm 79.7 ^b	161.2 \pm 13.1 ^a	1,283.7 \pm 205.0 ^b	96.7 \pm 8.0 ^a	6.3 \pm 0.9 ^b
Casein-based	78.9 \pm 7.7 ^{a,b}	5.3 \pm 0.1	13.9 \pm 0.5 ^a	46.0 \pm 2.0	820.0 \pm 26.5 ^a	164.7 \pm 14.4 ^a	1,683.6 \pm 52.1 ^a	96.3 \pm 6.7 ^a	8.6 \pm 0.3 ^a
Fish meal-based	71.1 \pm 8.4 ^b	5.5 \pm 0.2	11.7 \pm 0.3 ^b	46.0 \pm 3.5	307.0 \pm 6.1 ^c	111.2 \pm 12.1 ^b	569.6 \pm 46.0 ^c	68.1 \pm 5.0 ^b	2.9 \pm 0.1 ^c
Soybean meal-based	81.1 \pm 3.8 ^{a,b}	5.6 \pm 0.2	11.6 \pm 0.6 ^b	46.0 \pm 3.5	332.3 \pm 38.1 ^c	107.8 \pm 8.6 ^b	621.1 \pm 28.6 ^c	67.0 \pm 5.3 ^b	3.2 \pm 0.4 ^c
Rice bran-based	74.4 \pm 8.4 ^b	5.4 \pm 0.4	9.8 \pm 0.1 ^c	44.0 \pm 5.3	186.7 \pm 10.2 ^d	81.5 \pm 13.6 ^c	327.4 \pm 45.7 ^d	48.5 \pm 4.5 ^c	1.6 \pm 0.1 ^d

Means and standard deviations are presented ($n = 90$). Analysis of variance and Duncan's multiple range test were performed on the means of shell length and body weight increases; the same letter identifies the values that are not significantly different ($P < 0.05$).

gains corresponded to growth rates of 68.1 and 67.0 $\mu\text{m}/\text{day}$, respectively (Table 3). The lowest cumulative increase in shell length was found in abalone fed the rice bran-based diet (81.5%), which equated to a growth rate of 48.5 $\mu\text{m}/\text{day}$ (Fig. 1, Table 3).

Body Weight

The maximum cumulative weight increase (1683.6%) was found in the casein-based diet group that gave a growth rate of 8.6 mg/day (Fig. 2, Table 3). The control also had a high cumulative weight increase (1283.7%) and growth rate of 6.3 mg/day. Abalone fed soybean meal- and fish meal-based diets had lower cumulative increases in weight (621% and 569.6%, respectively) (Fig. 2, Table 3). These equated to growth rates of 3.2 and 2.9 mg/day, respectively (Table 3). The lowest cumulative increase in weight was found in abalone fed the rice bran-based diet (327.4%) that resulted in a growth rate of 1.6 mg/day.

Survival Rate

The survival rates of *H. asinina* fed different artificial diets and on algal control diet were significantly different ($P < 0.05$) (Fig. 3, Table 3). Two different groups could be recognized: (1) control (88.9%), soybean meal-based diet (81.1%), and casein-based diet (78.9%); (2) rice bran-based diet (74.4%) and fish meal-based diet (71.1%) groups.

Food Conversion Rate

The dry weight FCR was significantly different ($P < 0.05$) among four dietary groups (Table 4): (1) control (0.3); (2) casein-based diet (1.5); (3) soybean meal-based diet (4.0); (4) fish meal-based diet (6.1); and rice bran-based diet (6.4).

DISCUSSION

Early juvenile *H. asinina* provided *Acanthophora* sp. and a casein-based diet (as a main source of protein) produced superior growth rates (for shell length and body weight) and survival rates compared with juveniles fed fish meal-based, soybean meal-based, and rice bran-based diets. The growth and survival rates of the casein-based diet group were not significantly different from those fed macroalgae (control). The dietary value of casein-based diet was comparable to that of the macroalgae. The growth rates in shell length (96.8 $\mu\text{m}/\text{day}$ in the macroalgae group and 96.3 $\mu\text{m}/\text{day}$ in the casein-based diet group) are comparable with that reported by Kunavongdate et al. (1995), who found that juvenile *H. asinina* (31 mm in shell length) fed *Laurencia* sp. yielded a growth rate of 98 $\mu\text{m}/\text{day}$ (Table 5). Upatham et al. (1998) reported a lower growth rate (70 $\mu\text{m}/\text{day}$) in juvenile *H. asinina* (13 mm in shell length) fed *Gracilaria tenuistipitata*. In comparison, Krueatrachue et al. (2000) reported very low growth rates (29–36 $\mu\text{m}/\text{day}$) for juvenile *H. asinina* (13 mm in shell length) fed casein-based diets containing various species of red algae (Table 5). They

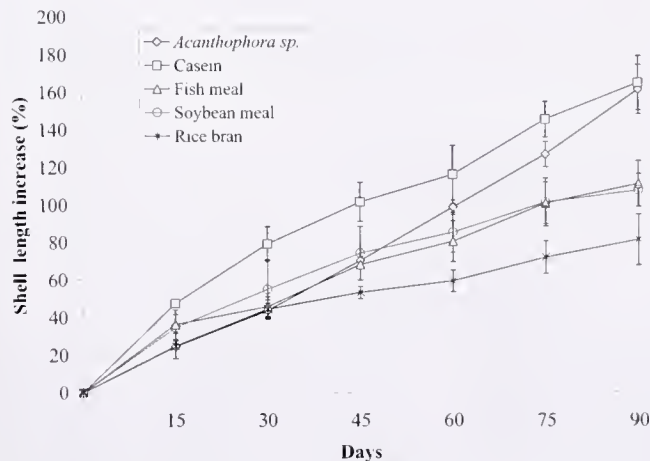


Figure 1. Percentage shell length increase of *H. asinina* fed artificial diets containing various protein sources over a period of 90 days. ($n = 90$, error bars are standard deviations, three replicates)

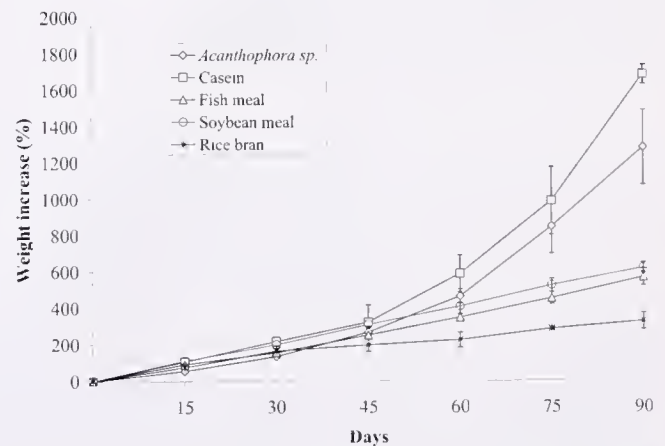


Figure 2. Percentage weight increase of *H. asinina* fed artificial diets containing various protein sources over a period of 90 days. ($n = 90$, error bars are standard deviations, three replicates)

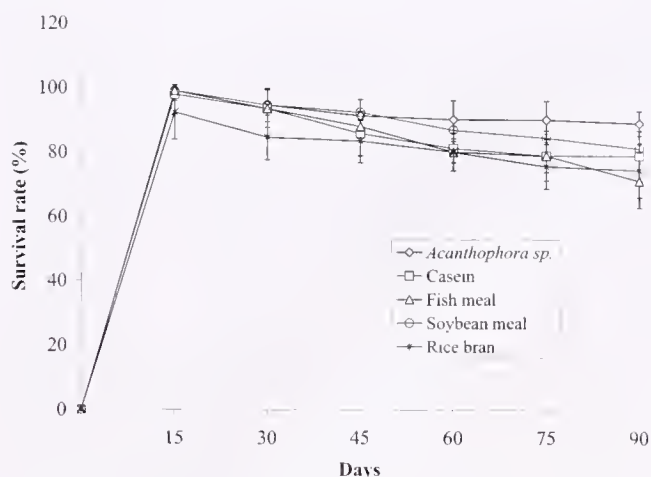


Figure 3. Percentage survival of *H. asinina* fed artificial diets containing various protein sources over a period of 90 days. ($n = 90$, error bars are standard deviations, three replicates)

suggested that these formulated diets were unsuitable and significant leaching of some trace nutrients might occur, resulting in poor growth rates (Kruatrachue et al. 2000). Jackson et al. (2001) studied the suitability of Australian formulated diets for aquaculture of *H. asinina* and found that a natural diet of *G. edulis* still produced the highest growth rate (56 $\mu\text{m/day}$; Table 5), while four formulated diets from two Australian abalone feed companies produced lower growth rates of 19–54 $\mu\text{m/day}$ (Table 5). However, the growth rates of *H. asinina* of our study were low when compared with the study of Capinpin & Corre (1996) who reported that the growth rates of juvenile *H. asinina* fed *G. heteroclada* and a commercial artificial diet were 193 and 192 $\mu\text{m/day}$, respectively (Table 5). Capinpin et al. (1999) also recorded a shell length growth rate of 164 $\mu\text{m/day}$ for *H. asinina* reared in sea cages and fed *G. bailinae*. Bautista-Teruel & Millamena (1999) achieved growth rates ranging from 222 to 247 $\mu\text{m/day}$ over 90 days when 20 juvenile *H. asinina* (18.3 mm in shell length) were housed in large 60-L containers and fed either of three formulated diets. These are the highest growth rates ever reported for *Haliotis* species (Table 5). In this study, *H. asinina* were reared in small (5-L) enclosures, which may have contributed significantly to the lower growth rates (49–96 $\mu\text{m/day}$) compared to other studies. The results are not directly comparable because of differing experimental conditions and the smaller size of juvenile *H. asinina* used in this

study. In general, certain species of red algae seemed to produce higher growth rates in *H. asinina* than artificial diets.

Table 5 also summarizes the reported data on growth rates of abalone fed artificial diets. Most studies evaluated the quality of several protein sources in diets such as casein, fishmeal, and soybean meal. A casein-based diet produced a higher growth rate (133 $\mu\text{m/day}$) in juvenile *H. discus hannai* (34 mm), compared with those fed either a soybean-based diet (106 $\mu\text{m/day}$) or a fishmeal-based diet (74 $\mu\text{m/day}$) for 40 days (Uki et al. 1985). Uki et al. (1986b) also reported a higher growth rate (82 $\mu\text{m/day}$) in juvenile *H. discus hannai* fed a casein-based diet than in those fed a fishmeal-based diet (47 $\mu\text{m/day}$), for 50 days. These reported results were in agreement with the results of this study on juvenile *H. asinina* (5–6 mm).

In comparison Britz (1996a, 1996b) reported that a fish meal-based diet produced a higher growth rate (65, 83–96 $\mu\text{m/day}$) than a casein-based diet (45 $\mu\text{m/day}$) in juvenile *H. midae* (20–21 mm) (Table 5). Similarly, Lopez et al. (1998) also reported that a fish meal-based diet resulted in a higher growth rate (135 $\mu\text{m/day}$) than a casein-based diet (119 $\mu\text{m/day}$) (Table 5) in *H. tuberculata* (3.2 mm). However, Viana et al. (1993) reported that a casein-based diet and a fish meal-based diet produced similar growth rates (98 $\mu\text{m/day}$ and 101 $\mu\text{m/day}$, respectively) in *H. fulgens* (13 mm) (Table 5). In *H. asinina* of this study, fish meal-based and soybean meal-based diets produced the second highest growth rates (68 $\mu\text{m/day}$ and 67 $\mu\text{m/day}$, respectively). The source and the processing of the fishmeal used in these studies may account for these differences. A soybean meal-based diet was also reported to produce high growth rates in *H. discus hannai* (106 $\mu\text{m/day}$) (Uki et al. 1985) and *H. diversicolor supertexta* (105–163 $\mu\text{m/day}$) (Chen & Lee 1999) (Table 5).

The most appropriate diet for *H. asinina* culture depends on the availability and cost of various protein sources and the growth rates produced. Although a casein-based diet produced the highest growth rate in this study, casein is unlikely to be widely used as a primary protein source in practical diets because of its high cost. Fishmeal is more appropriate since it is readily available and less expensive than casein. Even though a fish meal-based diet produced a lower growth rate than casein-based diet in *H. asinina*, in other *Haliotis* species it produced higher growth rates. There is, however, an increasing concern about the future supply and demand for fish meal, and this concern has led to efforts to reduce its use as the major protein source in commercial aquaculture feed formulations (Barlow 1989, Ramsey 1993). Because *Haliotis* are

TABLE 4.
Food conversion rate of *H. asinina* fed different diets over a period of 90 days.

Diet	Number and Total Wet Weight*				Total Wet Weight Gain (g)	Total Dry Weight Gain (g)	Dry Weight of Food Intake (g)	FCR
	Initial		Final					
	Number	Weight (g)	Number	Weight (g)				
Control	90	4.1 ± 0.4	80	49.6 ± 2.3	45.5 ± 2.7	28.9 ± 1.7	8.7 ± 0.0	0.3 ± 0.1 ^a
Casein-based	90	4.1 ± 0.2	71	58.2 ± 3.5	54.1 ± 1.4	35.2 ± 0.3	56.3 ± 0.2	1.5 ± 0.1 ^b
Fish meal-based	90	4.1 ± 0.3	64	19.8 ± 1.4	15.7 ± 0.5	9.9 ± 0.1	60.4 ± 0.6	6.1 ± 0.4 ^d
Soybean meal-based	90	4.1 ± 0.6	73	24.1 ± 2.8	20.0 ± 1.1	14.1 ± 0.7	56.4 ± 0.5	4.0 ± 0.4 ^c
Rice bran-based	90	3.9 ± 0.5	67	12.7 ± 0.5	8.8 ± 0.4	6.0 ± 0.3	38.4 ± 0.2	6.4 ± 1.1 ^d

Means and standard deviations are presented ($n = 90$). Analysis of variance and Duncan's multiple range test were performed on the means of growth rate; the same letter identifies the values that are not significantly different ($P < 0.05$); *three replicates.

TABLE 5.
Comparative growth of different species of *Haliotis* fed artificial diets and macroalgae.

Species	Size (mm)	Diet	Duration (day)	Growth Rate ($\mu\text{m}/\text{day}$)	FCR	Reference
<i>H. discus hannai</i>	34	Casein-based (30% protein)	40	133		Uki et al. (1985)
	24	Fish meal-based (32% protein)	40	74		
	34	Soybean meal-based (31% protein)	40	106		
	31	Casein-based (4.8–43.1% protein)	50	32–82		Uki et al. (1986b)
	31	Fish meal-based (5.6–43.1% protein)	50	32–47		
	7	Artificial diet	70	135		Nie et al. (1986)
	13	Artificial diet	70	101		
<i>H. fulgens</i>	21	Artificial diet	270	62		Corazani & Illanes (1998)
	13	Casein-based (44% protein)	90	98		Viana et al. (1993)
	13	Fish meal-based (35% protein)	90	101		
<i>H. midae</i>	17	Artificial diet	106	42	0.02	Serviere-Zaragoza et al. (2001)
	7.9	Artificial diet (35.5% protein)	30	59		Knauer et al. (1996)
	20	Fish meal-based (27–47% protein)	95	83–96		Britz (1996a)
	21	Casein-based (31% protein)	124	45	0.7	Britz (1996b)
	21	Fish meal-based (29% protein)	124	65	0.8	
	10.6	Fish meal-based (34% protein)	180	83	0.92	Shipton & Britz (2001)
	10.6	Fish meal/torula yeast	180	91	0.8	
	10.6	Fish meal/soy meal	180	85	0.82	
	10.6	Fish meal/sunflower meal	180	87	1.0	
	10.6	Fish meal/ <i>Spirulina</i>	180	82	0.97	
	10.6	Fish meal/corn gluten	180	70	0.98	
	26	Soybean-based (30% protein)	33	105–163		Chen & Lee (1999)
	26	Soybean-based (30% protein)	395	42–68	3.0	
<i>H. tuberculata</i>	3.2	Casein-based (36% protein)	105	119	0.8	Lopez et al. (1998)
	3.2	Fish meal-based	105	135	0.9	
<i>H. rufescens</i>	21	Artificial diet	270	45		Corazani & Illanes (1998)
<i>H. australis</i>	3–6	Artificial diet	365	40–47		Moss (1997)
<i>H. asinina</i>	28	<i>Gracilaria salicornia</i>	120	51	20.4	Singhagraiwan (1996b)
	31	<i>G. fisheri</i>	120	21	29.5	Kunavongdate et al. (1995)
	31	<i>Laurencia</i> sp.	120	98	18	
	15	<i>G. heteroclada</i>	90	193		Capinpin & Corre (1996)
	15	<i>Kappaphycus alvarezii</i>	90	59		
	15	Artificial diet (32% protein)	90	192		
	13	<i>G. tenuistipitata</i>	184	70	3.3	Upatham et al. (1998)
	13	<i>G. fisheri</i>	184	48	7.4	
	13	<i>G. salicornia</i>	184	59	15.5	
	13	<i>Acanthophora spicifera</i>	184	62	5.7	
	15	<i>G. bailinae</i>	90	164		Capinpin et al. (1999)
	18	Formulated diets	90	222–247	1.4–1.8	Bautista-Teruel & Millamena (1999)
	13	Casein + <i>G. tenuistipitata</i>	140	36	27.5	Kruatrachue et al. (2000)
	13	Casein + <i>G. fisheri</i>	140	36	29.8	
	13	Casein + <i>G. salicornia</i>	140	29	19.2	
	18	Artificial diets	168	19–54	1.4–1.8	Jackson et al. (2001)
	5.3–5.6	Casein-based (30% protein)	90	96	1.5	Present study
	5.3–5.6	Fish meal-based (30% protein)	90	68	6.1	
	5.3–5.6	Soybean meal-based (30% protein)	90	67	4.0	
	5.3–5.6	Rice bran-based (30% protein)	90	49	6.4	
	5.3–5.6	<i>Acanthophora</i> sp.	90	97	0.3	

naturally herbivorous animals with a digestive and enzymatic physiology equipped for processing plant materials. Shipton and Britz (2001) studied the partial and total replacement of fishmeal with selected plant protein sources (soy meal, sunflower meal, torula yeast, corn gluten, and *Spirulina*) in diets for *H. midae*, a

South African species. No significant differences were found in the growth rates between the control diet (100% fishmeal; 83 $\mu\text{m}/\text{day}$) and diets in which 30% of the fishmeal component was replaced by plant proteins (Table 5). Among the plant protein sources, fishmeal with torula yeast produced the highest growth rate (91

$\mu\text{m/day}$) (Table 5). The lowest growth rate was found in fishmeal and corn gluten (70 $\mu\text{m/day}$) (Table 5). Substitution of 50% fishmeal with either soy meal or *Spirulina* did not affect growth rates. Replacement of either 75% or 100% fishmeal with plant protein sources (sunflower meal, soy meal, and *Spirulina*) produced growth rates comparable to that of the control. Shipton and Britz (2001) concluded that there was a potential to replace fish meal with plant protein sources in commercial diets for *Haliotis*, especially considering the low raw material costs associated with soybean meal and sunflower meal. This study shows that for *H. asinina*, the soybean meal-based diet, yielded similar growth rates to fishmeal-based diet, and the survival rate was higher. Other factors also affect the growth rate of abalone, including water temperature, (Leighton 1974, Leighton et al. 1981, Uki et al. 1981, Hahn 1989, Lopez et al. 1998), the species of abalone (Uki & Kikuchi 1979), the rearing system (Moss 1997, Chen & Lee 1999) changing the diet of juveniles from diatoms to seaweeds, or artificial diets at the appropriate size. These factors will also affect the early growth of juvenile *H. asinina* and need to be investigated.

In general, abalone fed artificial diets have displayed higher rates of weight gain and length increase, and lower FCR compared with those fed macroalgae (Table 5). FCRs obtained for macroalgae have also differed among species (Table 5). *H. asinina* fed *G. tenuistipitata* and *Acanthophora spicifera* showed lower FCRs

(3.3 and 5.7, respectively) while those fed *G. salicornia*, *G. fisheri*, and *Laurencia* sp. showed higher FCRs (15.5 and 20.4, 7.4 and 29.5, and 18.0, respectively) (Singhagrawan 1991b, Kunavongdate et al. 1995, Upatham et al. 1998; Table 5). The FCRs of *H. asinina* fed casein-based diets containing various species of macroalgae were higher than those of macroalgae (Upatham et al. 1998, Kruatrachue et al. 2000; Table 5). In contrast, abalone fed artificial diets showed lower FCRs, for example, *H. tuberculata* (0.8–0.9; Lopez et al. 1998), *H. midae* (0.7–1.0; Britz 1996b, Shipton & Britz 2001), *H. diversicolor supertexta* (3.0; Chen & Lee 1999), *H. asinina* (1.4–1.8; Bautista-Teruel & Millamena 1999, Jackson et al. 2001; Table 5). In this study, other than the control (*Acanthophora* sp.), the FCR of the casein-based diet was low (1.5) and similar to those reported by Bautista-Teruel and Millamena (1999) and Jackson et al. (2001). Fleming et al. (1996) reviewed the formulated diets of temperate abalone offered by various feed manufacturers around the world and reported an FCR range of 0.77 to 3.33. The FCR value for the casein-based diet of this study compares favorably.

ACKNOWLEDGMENTS

This study was supported by the Thailand Research Fund BRG/04/2543.

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KARYOTYPE OF PACIFIC RED ABALONE *HALIOTIS RUFESCENS* (ARCHAEOGASTROPODA: HALIOTIDAE) USING IMAGE ANALYSIS

CRISTIAN GALLARDO-ESCARATE,^{1,2} JOSUÉ ÁLVAREZ-BORRERO,^{2,*}
MIGUEL ÁNGEL DEL RÍO PORTILLA,¹ AND VITALY KOBER³

¹Departamento de Acuicultura, División de Oceanología, ²Departamento de Óptica, ³Departamento de Ciencias de la Computación, División de Física Aplicada, Centro de Investigación Científica y de Educación Superior de Ensenada, Km 107 Carretera Tijuana – Ensenada, Código Postal 22860, Ensenada, B.C. México

ABSTRACT This report describes a karyotypic analysis in the Pacific red abalone *Haliotis rufescens* using image analysis. This is the first karyotype reported for this species. Chromosome number and karyotype are the basic information of a genome and important for ploidy manipulation, genomic analysis, and our understanding about chromosomal evolution. In this study we found that the diploid number of chromosomes in the red abalone was 36. Using image analysis by rank-order and digital morphologic filters, it was possible to determine total length of chromosomes and relative arm length in digitally enhanced image, elimination of noise and improving the contrast for the measurements. The karyotype consisted of eight pairs of metacentric chromosomes, eight pairs of submetacentric, one pair submetacentric/metacentric, and one pair of subtelocentric chromosomes. The black abalone, *Haliotis cracherodii*, also with 36 chromosomes and with a similar geographic distribution, has eight pairs of metacentric, eight pairs of submetacentric, and two pairs subtelocentric. This study contributes with new information about the karyology in the family Haliotidae found in California Coast waters and gives some support the Thetys' model about biogeographical origin, from the Mediterranean Sea to the East Pacific Ocean.

KEY WORDS: karyotype, *Haliotis rufescens*, chromosome number, image analysis, red abalone

INTRODUCTION

Abalones are economically important marine gastropods that reach moderate-to-high prices in the world market (Oakes & Ponte 1996). To date, only eight species of abalone, *Haliotis corrugata*, *H. cracherodii*, *H. fulgens*, *H. kamtschakana*, *H. kamtschakana*, *H. k. assimilis*, *H. sorenseni*, *H. walallensis*, and *H. rufescens*, have been reported in the further East Pacific (Geiger & Poppe 2000). The Pacific red abalone, *H. rufescens*, is found from southern Oregon to northern California in the United States and to the Baja California Peninsula in Mexico (Lindberg 1992). Aquaculture production of this species began during the last decade in Mexico, where the whole life cycle is managed under controlled conditions.

In addition to the importance on conservation, taxonomic work and karyological analyzes are also useful tools in providing fundamental information for animal breeding programs such as inter-specific hybridization (Miyaki et al. 1997) and chromosome-set manipulation (Arai et al. 1986, Fujino et al. 1987, Kudo et al. 1991, Zhang et al. 1998). A review about the biogeographical origin of the Family Haliotidae reported by Geiger & Groves (1999) using chromosome data in abalone showed that the abalones could be classified in three groups according to their chromosomes number. The first group ($2n = 28$), inhabits the European–Mediterranean area. The second ($2n = 32$) inhabits the Indigo–Pacific region, and the third group ($2n = 36$) represents the abalones of the North Pacific region. Geiger & Groves (1999) suggest that the biogeographical origin of abalone was in the European–Mediterranean area because *H. tuberculata* ($2n = 28$) is a relict species. Most probably this area could have been the starting point of the radiation, extending the distribution of the abalone forward to East from the Indo–Pacific to the North Pacific Ocean. This theory has been called the Thetys' model. In the California Coast, however, karyological data have been performed only for the black abalone *H. cracherodii* (Minkler, 1977). Thus, more studies are necessary to support the Thetys' model and to know the karyology of abalone species from California Coast.

In recent years, great attention has been given to image processing by the use of rank-order statistics. Rank-order and morphologic digital filters can be applied to computer and optical research (Kober et al. 2001a). These digital filters are very efficient in removing additive and impulsive noise, as well as in image enhancing and restoring the microscopical images. The reason for their success in image processing is that they can suppress noise without destroying important image details, such as edges and fines lines. The aim of this work is to describe the karyotype of Pacific red abalone *Haliotis rufescens* by using rank-order and morphologic filters for suppression of noise and enhancing contrast of the chromosomal images.

MATERIALS AND METHODS

Gametes

Pacific red abalone *Haliotis rufescens* were obtained from the broodstock kept in the commercial farm "Abulones Cultivados S.A" at Eréndira, Baja California, México. The abalones were maintained in a 250-L tank with a recirculating sea water system until the spawning induction. Gametes were obtained through induction spawning according to Morse et al. (1977). Eggs were collected in a 25- μ m sieve and resuspended into 2-L container with seawater filtered (0.45 μ m) and irradiated with ultraviolet light. Fertilization was conducted by adding sperm to the eggs suspension at a final density of 50 sperms per egg.

Chromosome Obtaining

Twenty to 24 hours after fertilization, swimming trochophore larvae of pacific red abalone were placed in a 0.005% colchicine solution for 3–4 h. After the antimitotic treatment, the samples were subjected to 45 min in a hypotonic solution (50% sea water and distilled water). The larvae were fixed in Carnoy solution (3:1, methanol: acetic acid) by rinsing the samples three times in the fixative at intervals of 10 min. Fixed cells were stored at 5°C overnight in a refrigerator. The sample of cellular suspension was made by cell dissociation in 50% acetic acid and strong agitation

*Corresponding author. E-mail: josue@cicese.mx

with a Pasteur pipette. The cellular suspension was then dropped onto a heated microscope slides (45°C) and air dried.

Chromosome Observation via Computer

The chromosomal images were obtained from unstained metaphases. The best 10 images were digitalized with a 24-bit video color frame grabber (model CG-7, Scion Corp.) using a phase-contrast microscopy (Zeiss Axiolab) equipped with a RGB color video camera (model 1040 COHU). To remove impulse noise as well as small undesirable details in chromosomal image, we use rank-order filters with spatially connected neighborhoods (Kober et al. 2001a, 2001b). These filters use spatial and rank information of spatially connected areas of the input image within a moving window to produce the output. The used rank algorithm

consists of two steps. Outliers and small details are first detected using spatial relationships between the color image components. Then, the detected pixels are replaced with the output of the vector median filter over a local spatially connected area excluding the outliers, while desired detail and noise-free pixels are left unaltered. The size of a moving window is 7×7 elements. The spatially connected neighborhood called as a *CEV* neighborhood is defined as a subset of pixels ($v_{n,m}$) of the moving window, which are spatially connected with the central pixel $v_{k,l}$, and whose values deviate from the value of the central pixel at most by predetermined quantities. The rank filter is given by:

$$\hat{v}_{n,m} = \begin{cases} v_{n,m}, & \text{if } \text{SIZE}(\text{CEV}[v_{n,m}]) \geq \text{Thrd} \\ \text{MED}(S[v_{n,m}] - \text{CEV}[v_{n,m}]), & \text{otherwise} \end{cases}$$

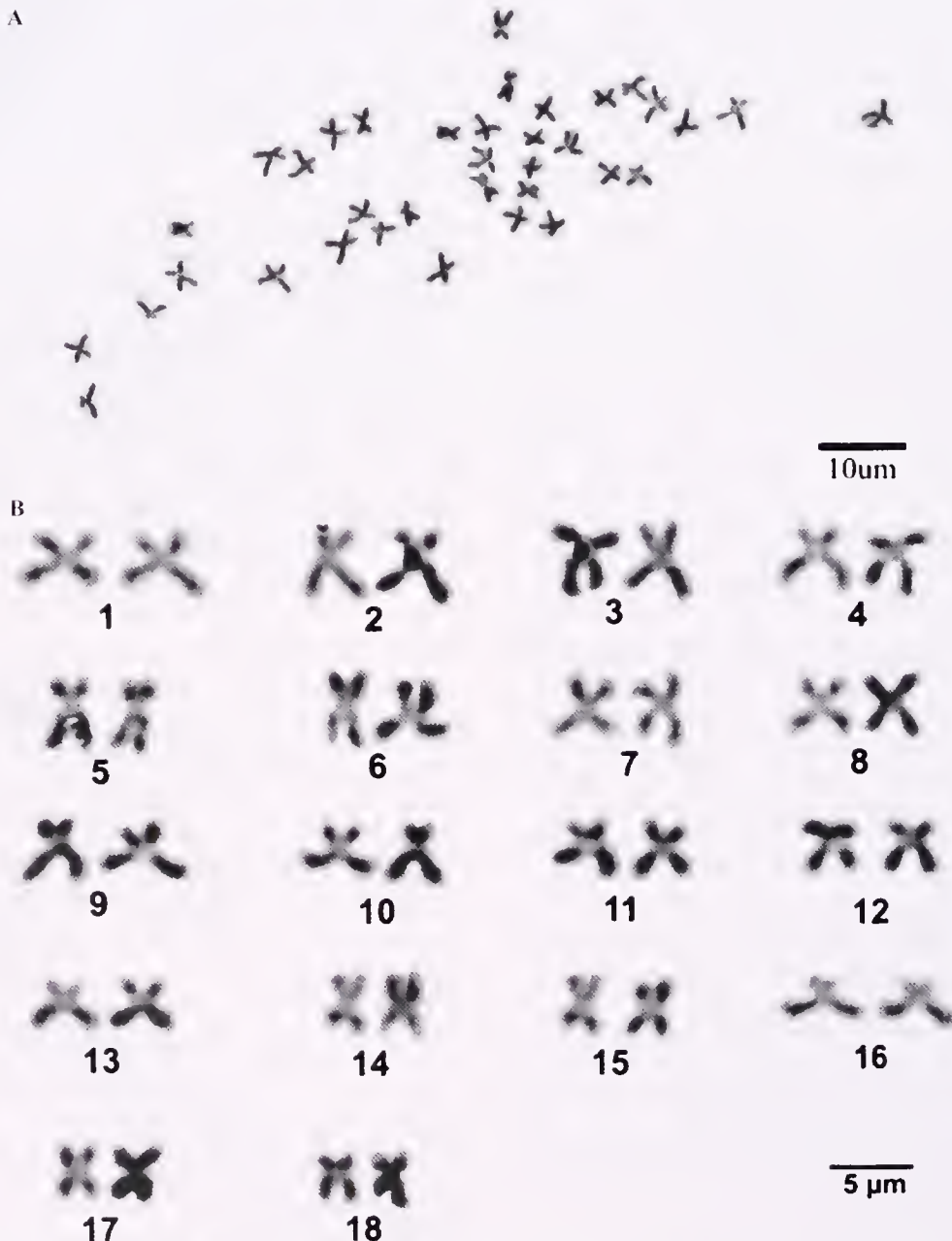


Figure 1. A, Digital image of metaphase plate from larvae of *Haliotis rufescens*. B, Karyotype of Pacific red abalone *Haliotis rufescens*.

where *Thrd* is a threshold value of either outlier or undesired detail detection, "-" denotes the set difference operation; *S* is a small subset of the pixels of the moving window. The pixels associated with outliers after the detection are excluded from the *S* neighborhood. *MED* and *SIZE* denote the median operation and the size of a set, respectively.

Identification of homologue chromosomes was performed by digital correlation. After identification of homologue chromosomes, determinations of chromosome arm lengths (short and large) were measured with an Image Pro-Plus software version 4.0 (Media Cybernetics). The measurements were used for determining relative lengths of chromosomes and centromeric index ($100 \times$ length of short arm/total chromosome length). The relative lengths of the chromosome arm was plotted the karyo-ideogram according to Spotorno (1985). Karyotype was arranged by decreasing size and classified according to Levan et al. (1964).

RESULTS

All chromosomal images in this study were processed using image analysis. The original digital images were obtained with the CCD camera, in this images were preset noise and contrast low, however, after digital image processing it is observed a good performance on image edge, enhanced contrast of the chromosomes and allowing measurements morphologic more exact. Figure 1A, shows the images enhanced of unstained metaphase plate by the algorithm.

The karyological observations on larvae of Pacific red abalone showed a diploid number equal to 36 chromosomes (Fig. 1A). The distribution of relative chromosome lengths observed was derived to perform the karyotype of Pacific red abalone *Haliotis rufescens* (Fig. 1B). The mean values and standard deviations of the relative lengths and total lengths of 18 chromosome pairs were estimated from arm length measurements in well-spread unstained metaphase plates from the larvae using chromosomal image analysis.

TABLE 1.

Relative lengths (LR), total lengths (TL), and centromeric index (CI) of chromosomes of *Haliotis rufescens*

Chromosome	LR (%)	TL (μ m)	CI	Type
1	6.52 \pm 0.56	5.67 \pm 0.19	43.95 \pm 0.15	M
2	6.42 \pm 1.03	5.58 \pm 0.07	32.53 \pm 0.18	SM
3	6.20 \pm 0.55	5.39 \pm 0.06	46.91 \pm 0.11	M
4	6.09 \pm 0.78	5.30 \pm 0.10	31.30 \pm 0.13	SM
5	6.03 \pm 0.91	5.24 \pm 0.01	29.91 \pm 0.09	SM
6	5.97 \pm 0.26	5.20 \pm 0.03	44.66 \pm 0.15	M
7	5.84 \pm 0.44	5.08 \pm 0.13	41.68 \pm 0.03	M
8	5.83 \pm 0.68	5.07 \pm 0.07	31.72 \pm 0.29	SM
9	5.74 \pm 1.01	4.99 \pm 0.07	26.38 \pm 0.15	SM
10	5.34 \pm 0.80	4.65 \pm 0.04	28.97 \pm 0.13	SM
11	5.32 \pm 0.70	4.63 \pm 0.09	29.70 \pm 0.13	SM
12	5.26 \pm 0.52	4.58 \pm 0.02	34.67 \pm 0.08	SM/M
13	5.17 \pm 0.78	4.50 \pm 0.04	27.59 \pm 0.17	SM
14	5.10 \pm 0.17	4.44 \pm 0.07	46.34 \pm 0.21	M
15	4.96 \pm 0.31	4.32 \pm 0.05	42.74 \pm 0.20	M
16	4.95 \pm 1.63	4.31 \pm 0.05	16.31 \pm 0.14	ST
17	4.45 \pm 0.27	4.46 \pm 0.06	45.56 \pm 0.15	M
18	4.31 \pm 0.34	3.75 \pm 0.07	44.48 \pm 0.19	M

M, metacentric; SM, submetacentric; ST, subtelocentric.

sis, the maximum length of the chromosomes was $5.67 \pm 0.56 \mu$ m and the minimum was $3.75 \pm 0.071 \mu$ m (Table 1). The ratio of the chromosomes arms plotted in the karyo-ideogram indicated that this species possessed eight metacentric pairs (chromosome Nos. 1, 3, 6, 7, 14, 15, 17, and 18), eight submetacentric pair (nos. 2, 4, 5, 8, 9, 10, 11 and 13), one pair (no. 12) was classified as submetacentric/metacentric chromosome and one pair of subtelocentric chromosome (no. 16). Chromosome pairs 14 and 17 were difficult to identify using only such morphologic characteristics as the relative length, because of their mutually overlapping standard deviations (Fig. 2).

DISCUSSION

To our knowledge, this is the first report of ploidy level and chromosome number in the Pacific red abalone *Haliotis rufescens*. Karyological studies of abalone species belonging to the North Oriental Pacific are scarce; in fact, only the karyotype of the black abalone *H. cracherodii* is described with $2n = 36$ (Minkler, 1977), since unknown the karyology of Pacific red abalone *H. rufescens* in the California Coast at present. In contrast, karyological studies in abalones species have been reported in North Occidental Pacific (Arai et al. 1982, Nakamura 1986, Okumura et al. 1999). The morphologic comparison between *H. rufescens* and *H. cracherodii* showed that both species comprised 36 chromosomes. However, they are only similar in the metacentric chromosomes number equal to eight pairs. The differences are that red abalone has nine submetacentric pairs (chromosome no. 12, which is submetacentric/metacentric) and one subtelocentric pair, whereas the black abalone has eight submetacentric pairs and two subtelocentric pairs. This conformation of the karyotype in *H. cracherodii* suggest a deletion process of the short arm in one of submetacentric chromosome pairs, originating an extra subtelocentric pair. It is possible that *H. cracherodii* was divergent from the one species with more number of submetacentric chromosomes. This suggests that *H. cracherodii* has a higher genetic difference than *H. rufescens*. According to Owen et al. (1971) and Leighton (2000), relatively few cases of hybridization in mollusks have been reported, however, in *Haliotis*, with the exception of *H. cracherodii*, each southern Californian species of abalones was found to have hybridized with at least other species. The impossibility the *H. cracherodii* for hybridize with other species of abalones proved genetic distantly related.

Phylogenetic relationships studies by Meyer (1967) among California species were analyzed by immunochemical comparing of the hemocyanins and showed that phylogenetically close species were distinguished from those more distantly related. Two relatively closely related groups were found; 1) *H. rufescens*, *H. sorenseni*, and *H. kamtschaticana assimilis*, and 2) *H. corrugata*, *H. fulgens*, and *H. walallensis* appeared moderately distinct from these groups, whereas *H. cracherodii* was decidedly different from the other species. The amino acid sequences of lysin proteins of seven California abalone species were deduced from the cDNA sequences (Lee & Vacquier 1992, 1995). These studies are in general agreement with the immunochemical comparison by Meyer (1967), but *H. cracherodii* was placed closer to *H. corrugata*, leaving *H. fulgens* in a more distant relationship to the other California species.

Morphometric comparison of chromosomes between the other studied abalone species showed variations of diploid number

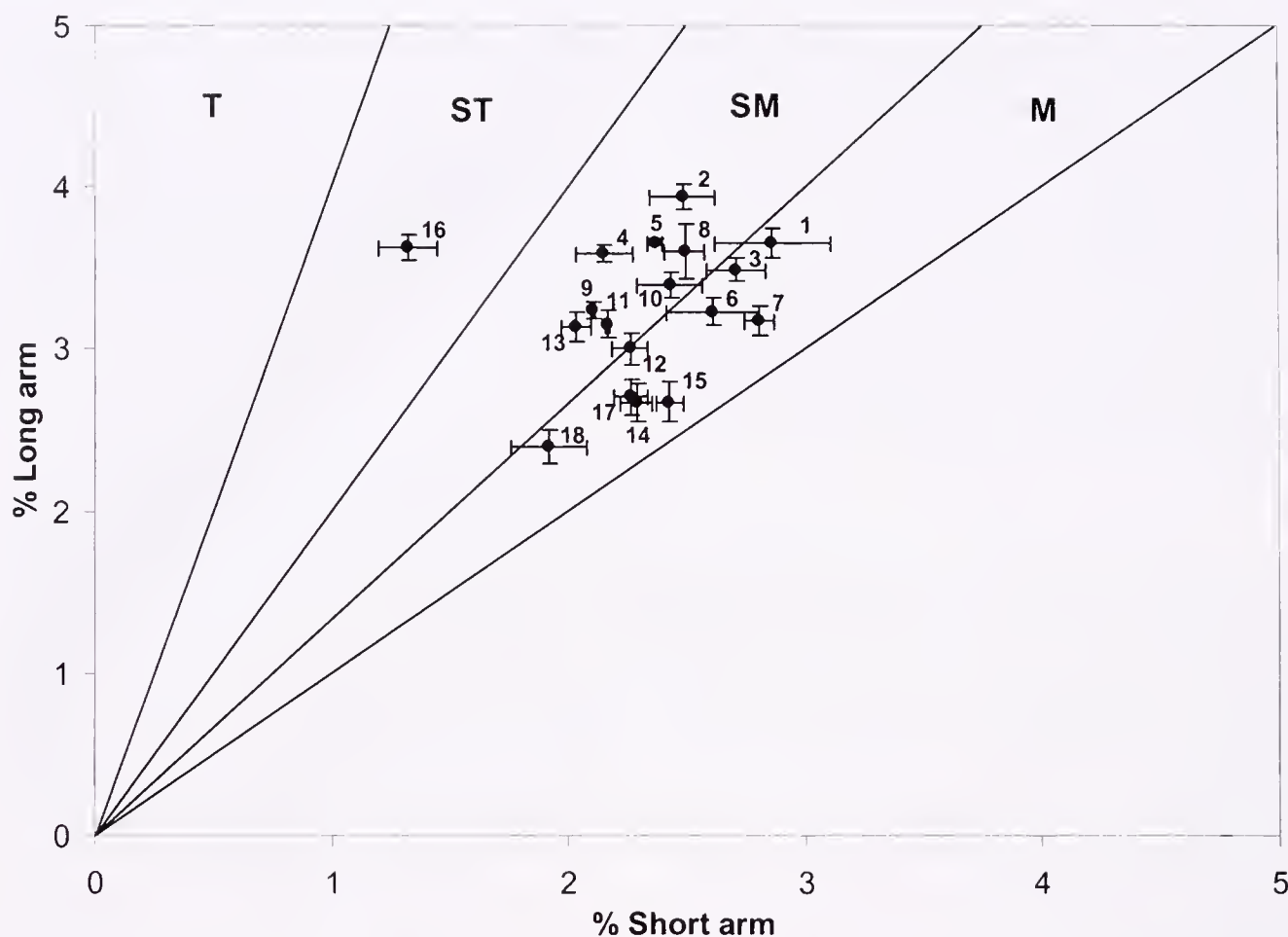


Figure 2. Karyo-ideogram of *Haliotis rufescens*. M, metacentric; SM, submetacentric; ST, subtelocentric; T, telocentric.

among 28 to 36 chromosomes (Jarayabhand et al. 1998) (Table 2). The review of diploid chromosome number of the existing karyotypes abalone according to Geiger & Groves (1999), showed that the increment begins in European Mediterranean area with $2n =$

28, Indo-Pacific region with $2n = 32$ characteristic, except for *H. aquatilis* with diploid number equal to 34 chromosomes, south Japan area with $2n = 32$ and finally Pacific North area with a chromosome number of 36.

TABLE 2.

Review of diploid chromosome number of abalone.

<i>Haliotis</i>	Diploid Number	Geographic Occurrence ^a	Reference
<i>H. tuberculata</i>	28	European-Mediterranean	Arai & Wilkins 1986
<i>H. lamellose</i>	32	European-Mediterranean	Colomera & Tagliaferri 1983
<i>H. diversicolor aquatilis</i>	32	Indo-Pacific	Nakamura 1985
<i>H. aquatilis</i>	34	Indo-Pacific	Nakamura 1985
<i>H. diversicolor</i>	32	Indo-Pacific	Arai et al. 1988
<i>H. planata</i>	32	Indo-Pacific	Arai et al. 1988
<i>H. varia</i>	32	Indo-Pacific	Jarayabhand et al. 1998
<i>H. asinina</i>	32	Indo-Pacific	Jarayabhand et al. 1998
<i>H. ovina</i>	32	Indo-Pacific	Jarayabhand et al. 1998
<i>H. exigua</i>	32	South Japan	Arai et al. 1988
<i>H. discus discus</i>	36	North Pacific	Arai et al. 1982
<i>H. discus hamai</i>	36	North Pacific	Arai et al. 1982, Okumura et al. 1999
<i>H. modaka</i>	36	North Pacific	Nakamura 1986
<i>H. cracherodii</i>	36	North Pacific	Winkler 1977
<i>H. rufescens</i>	36	North Pacific	This study

^a Geographical areas according Geiger & Groves 1999.

This study contributed with new information about the karyology in the family Haliotidae found in California Cost waters and supports the Thetys' model about biogeographical origin discussed by Geiger & Groves (1999) and Geiger & Poppe (2000).

ACKNOWLEDGMENTS

This study was supported by the Mexican National Council of Science and Technology (Project 36075-B). The authors thank to C. Paniagua for manuscript review and for technical assistance.

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PLICOPURPURA PANSA (GOULD, 1853) FROM THE PACIFIC COAST OF MEXICO AND CENTRAL AMERICA: A TRADITIONAL SOURCE OF TYRIAN PURPLE

LUDWIG C. A. NAEDEL*

Centro Interdisciplinario de Ciencias Marinas, Instituto Politécnico Nacional (CICIMAR/IPN), La Paz, B.C.S. 23000, México

ABSTRACT Most marine snails of the families Muricidae and Thaididae, which make up the genera *Murex*, *Thais*, and *Plicopurpura*, produce in the hypobranchial gland (mucus gland) a viscous liquid secretion. The secretion contains, besides mucus and biologically active compounds, minute amounts of chromogens, which develop enzymatically and under the influence of light and oxygen into a purple pigment known as “Tyrian purple,” “Royal purple,” or “Shellfish purple.” Throughout history, humans have used the pigment for various purposes. On the Pacific Coast of Mexico and Central America, for at least 500 y, the dyeing properties of the “snail’s ink” have been known and the pigments used for ceremonial and funeral garments. Here, the muricid *Plicopurpura pansa* (Gould, 1853) is the most exploited, and rarely the thaid *Thais kioskiformis*. *T. kioskiformis* has to be killed to obtain a few drops of the secretion from the hypobranchial gland, in contrast to *P. pansa*, which can be “milked” periodically without harming the animal to obtain a few milliliters of mucus containing the “Tyrian purple” precursors. In contrast to the Mediterranean region, where the use of purple from marine snails has long been forgotten and the craft of dyeing today cannot exactly be reconstructed, in remote Pacific regions of Mexico (in the states of Oaxaca and Michoacán) and in the Indian community of the Borucas in Costa Rica, its use continues until the present day on a small-scale and represents the survival of a knowledge of considerable antiquity. However, it is feared that this old tradition will be lost in the near future. The scarcity of the snails, the time, patience, and labor required for collecting them; and the great numbers of them required to dye a small piece of material are the main reasons why cheap, synthetic pigments are today used for dyeing traditional dresses.

KEY WORDS: *Plicopurpura pansa*, purple snail, Tyrian purple

INTRODUCTION

A large number of publications exist about the use of shellfish purple as a dye, mainly describing the source from Mediterranean snail species. Recently described was the source and chemistry of Tyrian purple from the west Pacific marine snail species *Plicopurpura pansa* (Naegel & Cooksey 2002). General references about the use of Shellfish purple are the books by Schweppe (1993) and Cardon (2003).

The majority of purple-producing marine snails belong to the family of Muricidae and most, if not all, produce a colorless secretion in the hypobranchial gland (also called mucus gland), which turns purple on exposure to air and light (Fretter & Graham 1994), giving what is known as “Tyrian purple,” “Royal purple,” or “Shellfish purple.” Mollusk purple as such does not occur in the live animal, but is formed during a sequence of chemical reactions in the presence of oxygen and light from the secretions produced by the animal. As in antiquity in the Old World, the use of muricids for dyeing led also on the Pacific Coast of the Americas to a product of high economic value. However, in contrast to the Mediterranean region, where the use of purple from marine snails has long been forgotten and the craft of dyeing today cannot exactly be reconstructed, in remote Pacific regions of Mexico (in the states of Oaxaca and Michoacán) and in the Indian community of the Borucas in Costa Rica, its use on a small-scale continues until today and represents the survival of a knowledge of considerable age (Turok 1999). As in the Old World, the conservative Indian communities in Mexico are convinced that Tyrian purple possesses properties that cannot be duplicated, apart from the practical advantage that material dyed with purple does not bleach. Cheap and fast synthetic dyes of purple color are today available, but the communities still prefer the expensive snail-purple. The garlic-like smell of material dyed with snail-purple, which aroma fades only after

several washings, is valued as a proof of authenticity (Gerhard 1962). Additionally, in various parts of the Americas, shellfish purple has long been used in the bridal dress of Indian women. Here, we may have an explanation for the great esteem in which shellfish coloring has been held on this continent, and consequently an analogy with the Old World belief that the purple from snails helps a woman to become prolific. There is also the possibility of a similar early idea that dye mollusks give protection from evil spirits or that shellfish colors are in some long-forgotten way connected with power, royalty, and religion (Gerhard 1962, Yoshioka 1974).

The use of muricids for dyeing cotton on the Pacific Coasts of Mexico and of Central America dates at least from Columbian times. Here, mainly *Plicopurpura pansa* (Gould, 1853) is exploited for Tyrian purple production. The carnivorous, gonochoristic marine snail inhabits intertidal rocks exposed to the open sea with high impact waves. The range of *P. pansa* extends at the Pacific from the northwest coast of Mexico (Baja California Sur) (Clench 1947; Keen 1971) to northern Peru (Peña 1970, Paredes et al. 1999). At low tides the mollusk is relatively easily gathered, and it ejects its dye-producing liquid in such a quantity that there is no need to kill the animal to obtain the purple. Furthermore, the dye-producing hypobranchial gland is so active that the snails can be “milked” periodically. The secretion is a milky-white liquid, which turns on exposure to air and light, at first yellow, then greenish, bluish, and finally purple (“Tyrian purple”).

The knowledge about the use of Tyrian purple on the Pacific side of Central America and Mexico before Columbus is very limited. Because of the tropical humid climate, the preservation of textiles is poor, and because the purple from *P. pansa* can be obtained without the need to kill the animal, shellfish middens are not found, as in Mediterranean and South American locations. However, we have written evidence of this ancient industry from several places in Central America, especially from the 17th and 18th centuries.

*Corresponding author. E-mail: lnaegel@cibnor.mx

HISTORICAL WRITTEN REPORTS ABOUT THE USE OF TYRIAN PURPLE

Possibly the first written report about the traditional use of Tyrian purple in Central America comes from the preacher Thomas Gage of England. Gage writes: "About Chira, Golfo de Salinas and Nicoya [Costa Rica] there are some farms of Spaniards, few and very small Indian towns who are all like slaves used by the Alcalde Major, to make him a kind of thread called pita [pita is made of agave fiber], which is a very rich commodity in Spain, especially of that color wherewith it is dyed in these parts of Nicoya, which is a purple color, for which the Indians are here much charged with work about the seashore, and there to find out certain shells, wherewith they make this purple dye. *Purpura* is a kind of shell-fish whose usual length of life is seven years. He hides himself about the rising of the Dog-star and so continues for 300 days. It is gathered in the spring time and by a mutual rubbing of one or the other of them together, they yield a kind of thick slime like soft wax, but its famous dye for garments is in the mouth of the fish and the most refined juice is in a white vein. The rest of his body is void and of no use. Your Segovia cloth dyed therewith for the richness of the color is sold at five or six pounds the yard, and used only by the greatest Dons of Spain [most probably Spaniards living in the Americas, since during this time Tyrian Purple was not used any more in Europe] and in ancient times only worn by the noblest Romans and called by the name of Tyrian purple. There are also shells for other colors, which are not known to be so plentiful in any other place as here." (Gage 1655).

Unfortunately, Thomas Gage did not describe in more detail which other gastropods were exploited for their Tyrian purple. The literature reports that related species of muricids produce various shades of purple, depending on the number and concentration of different chromogens, and the quantity of liquid produced varies greatly from one species to the next. Additionally, at certain seasons, the mucus gland (hypobranchial gland) is more active and its secretion is altered (Born 1936; Clench 1947; Gerhard, 1962).

Probably because of the over-exploitation of *P. pansa*, it became in Nicoya increasingly difficult to collect snails for dying, which caused increasing tensions between the gold-hungry Spanish magistrate and the indigenous population. The tensions exploded in 1760 in an uprising by the Indians (Fernández-Guardia 1938).

In 1744, during cartographic research, the Spanish scientists Jorge and Antonio Ulloa observed at Santa Elena close to Guayaquil in Ecuador, and also in Nicoya, the production and use of Tyrian purple from marine snails. Antonio Ulloa (1748) reports: "On the coasts belonging to the province of Guayaquil the finest purple is found. The animals from which it is derived are contained in shells, about the size of walnuts, and live on rocks washed by the sea. They contain a juice or humor, which is taken out, and yields the true purple. . . . Cotton, thread, and other delicate materials are dyed with it. It gives a lively and durable color, which does not lose its luster by frequent washing, but is rather improved thereby, and does not fade through long-continued use and exposure. Near the port of Nicoya the same kind of shellfish is found, and used for dyeing cotton. In both places the dyed yarn is used in making ribbons, lace, and other articles, which are afterwards skillfully sewn and embroidered. All such articles are highly prized on account of their fine and rare color. Various processes are used for extracting the juice or humor. Some kill the animal. They take it out of its shell, and having laid it on the back of the hand, press and

squeeze it with a knife from the head to the tail, concentrating the fluid in the "tail", which was finally cut off, the rest of the animal matter being thrown away. They treat in this way a number of animals until they have a sufficient quantity of juice. They then draw through the thread, which they wish to dye, and no more is required. But the dyed threads do not show at once the purple color they should have. This is not perceived until the whole is thoroughly dry. At first the color is milk-white, then it becomes green and finally purple. Others express the juice without killing the animal. They do not take it entirely out of the shell, but only press it as to cause a certain quantity of humor to be ejected, with which the threads are dyed. The shells are then laid again on the stones from which they were taken. They recover, and after some time give a fresh quantity of juice, but not so much as the first time. If the operation is repeated three or four times, the quantity is very small and the animal dies of exhaustion."

Ulloa continues the description of the dyeing activity in Nicoya: "a group of dyers would spend a month working from one bay to the next, and return during the following moon to "milk" the same animals again. One man could dye as much as a quarter of a pound of yarn in a single tide, or in theory 15 pounds a month. However, little work was done for a week or so near new moon, when it was thought that the dye was too thin or too meagre. It was believed that both the weight and color of shellfish-dyed thread varied according to the time of day, and sellers and buyers agreed upon a specific hour for their transactions."

It is not clear why the dyers found it either convenient or necessary to kill the animals to extract their color. *P. pansa* can be milked periodically without harming the animal. Perhaps of over-exploitation this species became rare, and therefore the dyers had to look for another purple-producing snail. The species of dye mollusk used toward the end of 1800 was probably *Thais kiosquiformis*. The animal was pricked with a needle or cactus thorn and then pressed down into his shell until it reluctantly yielded a few drops. The cotton threads were soaked and put into a bowl with more cotton into which the dye penetrated (Gerhard 1962).

About the process of purple dyeing as practiced in more recent times by the indigenous communities of Nicaragua, Squier (1852) gives us the following account: "Some of the cotton fabrics manufactured by the Indians are very durable and woven in tasteful figures and various colors. The color most valued is the Tyrian purple, obtained from the murex shellfish, which is found on the Pacific coast of Nicaragua. This color is produced of any desirable depth and tone, and is permanent, unaffected alike by exposure to the sun and to the action of alkalis. The process of dyeing the thread illustrates the patient assiduity of the Indians. It is taken to the seaside, when a sufficient number of shells are collected, which being dried from the sea water, the work is commenced. Each shell is taken up singly, and a slight pressure upon the valve which closes its mouth forces out a few drops of the coloring fluid, which is then almost destitute of color. In this each thread is dipped singly, and after absorbing enough of the precious liquid is carefully drawn out between the thumb and finger, and laid aside to dry. Whole days and nights are spent in this tedious process, until the work is completed. At first the thread is of a dull blue color, but upon exposure to the atmosphere acquires the desired tint. The fish is not destroyed by the operation, but is returned to the sea, where it lays in a new stock of coloring matter for a future occasion."

Apart from aspects of conservation, the "milking" of snails has the advantage that a dye can be obtained without interfering foreign substances.

TYRIAN PURPLE AS A COLORANT IN THE CODEx NUTTALL

In antiquity and in medieval times, Tyrian purple was used in special manuscripts ("Purple codices") as a priming color of parchments. In addition, in a pre-Columbian Mixtec (Codex Nuttall) manuscript, Tyrian purple was applied from an as-yet undetermined snail source, however not as priming color, but as paint. The Codex Nuttall was produced between 1468 and 1519 and describes the wars of conquest of powerful armies, which came to the south of Mexico. Distinguished ladies dressed in purple skirts, ponchos, and coats are shown stained with the same color, and chiefs wearing a purple apron and headdresses. Additionally in this manuscript, it is shown that purple was probably used as a body colorant for priests (Turok et al. 1988).

TEXTILES DYED WITH TYRIAN PURPLE

Due to the humid tropical climate in Mexico and Central America, the conditions for textile conservation are poor. However, about fifty years ago, Mayan textile fragments sealed in a jar were found in the state of Chiapas, Mexico. One band of textile was painted purple with a brush on both sides, most probably from *P. pansa*. It is not possible to date these textiles accurately, however, it is believed that the association of textiles together with charred human bones found in sealed jars belongs to a period not long before the Spanish conquest, and possibly immediately after it (Johnson 1954).

ABUNDANCE OF PURPLE SNAILS IN THE PAST

Undisputed evidence on the use of Tyrian purple on the Pacific coast of Central America and Mexico before the arrival of Spaniards is missing. During the Spanish time, three regions of shellfish dyeing could be found: Veraguas (Panama), the peninsula of Nicoya (Costa Rica), and Oaxaca (Mexico). However, it is not clear which exact species of purple snail were exploited in these regions, but most probably *P. pansa* and in cases of over-exploitation also *T. kioskiformis*.

The peninsula of Nicoya (Costa Rica) was the center for purple production, and materials dyed with purple were exported to Guatemala and Panama. The mayor of Nicoya had exclusive rights for gathering and distributing dyed skeins in Central and South America. The over-exploitation of the purple snails resulted in a reduction of the snail population, similar to what had happened centuries earlier with Tyrian purple in the Mediterranean (Turok and Acevedo 2000). For this reason, the purple industry in Nicoya ceased to exist by the end of the 19th century, and not for a scarcity of dyers or a reduced demand for the product.

About 1880, the English chemist Schunck (1880) received from the west coast of Nicaragua cotton yarn dyed with the ink from *P. pansa*. It is interesting to note his remark that the animals were extracted, as in Ecuador 150 y earlier, from their shells by means of a pin or other pointed instrument, and the blood furnished the purple dye. This way of obtaining the "ink" certainly drastically reduced the snail population.

The use of "shellfish dye" in Mexico was at first reported in 1874 by von Martens (1874). He obtained from Tehuantepec (Oaxaca) a purple-dyed skirt and material with woven-in purple stripes. He mentioned that the high value of purple-dyed "enagua" skirts can be explained by the high number of snails needed, which are not numerous there (von Martens, 1898). In 1909, the ethnologist Zelia Nuttall visited Tehuantepec, and she also reported that the population of the purple snails seemed to be over-exploited and

became scarce, in spite of the careful treatment of the snails during the "ink" collection. Fishermen started to collect purple at Huamelula, and due to the rarity of the snails, to fill their orders they were often forced to proceed as far north as Huatulco—about 100 km along the coastline away from Huamelula—or even to Acapulco (more than 500 km along the coastline from Huamelula). Nuttall continues, "The scarcity of the fish (snails), the great numbers of them required to dye a pound of cotton, and the time, patience, and labor required, amply account for the comparatively high price of three to five gold dollars for one hand-woven skirt (1909).

TYRIAN PURPLE USE IN MODERN TIMES

Nuttall (1909) regrets that fewer and fewer purple skirts are ordered every year, and that the younger generation of women favors the imported and cheaper European stuffs. At Tehuantepec, the modern center for using Tyrian purple, in 1908 not more than about 20 purple garments were woven, and Nuttall predicted that not before long the industry will be extinct. Nearly eighty years later, Thompson (1994) observed that "in the early 20th century in Mexico shellfish purple was in much more widespread use than it is now. The beliefs, languages, and crafts of the Mexican Indians are fast disappearing. The progressive "westernization" of rural Mexico has led people in many villages to abandon their traditional textiles and customs, in favor of factory-made cloth and western-style clothes which are readily available everywhere. Cultural and social decay is continuing to the point that the demand for traditional textiles has almost vanished. Weavers in a few villages formerly noted for their excellent textiles have turned to making more "commercial" articles for sale to people such as tourists, outside their culture—a classic manifestation of the "airport art" phenomenon."

Today only in remote Pacific regions of Mexico (in the states of Oaxaca and Michoacan) and in the conservative Indian communities of the Borucas in Costa Rica has the tradition of dyeing material with the colorant of purple snails survived (Turok 1999). The Borucas are still exploiting *P. pansa* and *T. kioskiformis* in the traditional way and are using the dye to make designs on woven fabrics (Gerhard 1962).

Today it is relatively easy to produce synthetically 6,6'-dibromoindigo; however, the interest in it is so small that it is not produced commercially (Imming et al. 2001). It can be considered that the market for a special color can change decisively, not due to technical or scientific influences, but due to historical and cultural perceptions. Tyrian purple has experienced changes during the centuries, not only in the technical, but also in cultural views. Originally, it was the sign of wealth and dignity, the symbol of the ruling class, and later as a high position in the church; today these perceptions have changed completely.

In recent years, however, with the gaining of importance of natural colors, the commercial exploitation of the purple snail for dyeing kimonos with natural "Tyrian purple" had reached in Mexico such levels as to threaten the survival of the species. In 1988, *P. pansa* had to be declared a species under special protection by the Mexican government (Anonymous 1988, Anonymous 1994).

P. pansa grows astonishingly slowly (Ramírez-Rodríguez & Naegel 2003). An animal with a total shell length of about 3 cm might be three years old, if not older. From snails of less than 2-cm shell length can be obtained 0.5 mL of secretion and from large

animals of 5–6 cm up to 4 mL (Rios-Jara et al. 1994). It has to be kept in mind, however, that of this volume only a minute proportion consists of the dye precursors. For this reason, it does not astonish that the enormous number of at least 1000 animals has to be “milked” to dye sufficient cotton threads for just three traditional skirts. In view of the slow growth of *P. pansa*, the small amount of colorant excreted, and the large number of snails needed for dyeing just one skirt, it was the right decision of the Mexican government to permit only Indian communities the traditional exploitation of *P. pansa* for its pigments and to declare it a species under special protection. One recent interesting development in Mexico has to be mentioned, which protect the purple snail on one side, and on the other side promote the traditional use of Tyrian purple and offer to the traditional dyers and weavers an income. Multidisciplinary groups, including indigenous Mixtec, Nahoá, and Boruca communities, social and scientific researchers, groups

of dyers and weavers, local, state, and federal authorities, ecologists, fishermen, and the tourism industry have all joined hands formulating an agreement that describes standards for the sustainable use of *P. pansa* and to create a natural protected area with limited access rights to the resource and promoting the snail culture and species conservation for the long-term (Turok and Acevedo 2000).

ACKNOWLEDGMENTS

I would like to thank Chris Cooksey (London) and Ran Boytner (Los Angeles) for their help in digging out old literature and for making suggestions to improve earlier versions of this manuscript. The anonymous reviewers helped with their suggestions to improve the article. Thanks to the Instituto Politécnico Nacional (COFAA and EDI) and the Consejo Nacional de Ciencia y Tecnología for their financial support.

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STUDY ON HERITABILITY OF GROWTH IN THE JUVENILE SEA URCHIN, *STRONGYLOCENTROTUS NUDUS*

LIU XIAOLIN,^{1,3} CHANG YAQING,² XIANG JIANHAI,^{1,*} DING JUN,² AND CAO XUEBIN²

¹Experimental Marine Biology Laboratory, Institute of Oceanology, CAS, Qingdao 266071, China; ²Key Lab of Mariculture and Biotechnology of the Ministry of Agriculture, Dalian Fisheries University, Dalian 116023, China; ³College of Animal Science and Technology, Northwest Sci-Tech University of Agriculture and Forestry, Yangling, Shaanxi 712100, China

ABSTRACT The heritability of growth of juvenile *Strongylocentrotus nudus* was analyzed using quantitative genetic methods. Twenty-one half-sib groups and 60–63 full-sib groups of juveniles were obtained by artificial fertilization of 3–5 females by single males based on a nested design. The body weight (g) and test diameter (cm) of the young were measured 3 and 5 mo after metamorphosis. Maternal component estimates are significantly greater than paternal component estimates for both weight and diameter at both ages. Greater maternal components suggest large nonadditive genetic effects that could not be differentiated with the available data. Estimates of heritability in the narrow sense calculated from the additive genetic component using a paternal half-sib correlation analysis ranged from 0.2167 to 0.4565 for weight and 0.2059 to 0.4998 for diameter. The results indicate significant maternal effects. The strength of the nested design and the paternal half-sib correlation analysis used in this study make the estimate the most precise and unbiased reported to date.

KEY WORDS: growth, heritability, sea urchin, *Strongylocentrotus nudus*

INTRODUCTION

Sea urchins are one of the most important aquaculture species in the world. The gonads have long been used as a luxury food and as a food source by common people in many countries (Hagen 1996, Hobson et al. 1990, Shimabukuro 1991). Because of over-fishing, interest in aquaculture of sea urchins has greatly increased (Hagen 1998, Lawrence 2001). One of the most important species for aquaculture is *Strongylocentrotus nudus* (Agatsuma 1998, Hagen 1996). Current culture of *S. nudus* has used seeds obtained from wild individuals (Gao and Chang 1999, Liao and Qiu 1999). Analysis of populations of *S. nudus* in Japanese waters show considerable range in size of small individuals, presumably of single cohorts (Agatsuma 1997). This could result from interaction of genetic characteristics and the environment. Vadas et al. (2002) found evidence for intrinsic variability in field populations of *Strongylocentrotus droebachiensis*. It is important to document the degree of heritability of growth in sea urchins because of its implications for both fisheries and aquaculture.

Demonstration of heritability is best done with comparisons of half-sib groups because they are less likely to be affected by environmental influence (Gjedrem 1992). Sib analysis techniques have been used for a variety of important aquaculture species (Benzie et al. 1997, Crenshaw et al. 1991, Hadley et al. 1991, Newkirk et al. 1977, Mallet et al. 1986, Rawson and Hilbish 1990). The purpose of the current study was to estimate heritability of growth in terms of body weight and diameter of juvenile *S. nudus*.

MATERIALS AND METHODS

Experimental Design

This study used a classic nested mating design developed by Comstock and Robinson (1952) to partition the phenotypic variation in juvenile growth into its genetic and nongenetic causes. In this experiment, each of 21 male *S. nudus* was mated to 3–5 females, therefore generating 63 full-sib families and 21 half-sib

families. The effects of males and females nested within males on growth were separated using nested analysis of variance (ANOVA). Juveniles were weighed and their diameters measured at 3 and 5 mo of age.

Genetic Analysis

The covariance among full- and half-sibs provides the basis for the separation of phenotypic variance into genetic and environmental components of variance. The covariance among full- and half-sibs are calculated from the observed components of variance obtained from a three-level nested, unbalanced ANOVA (Table 1) and the General Linear Models procedure of the Statistical Analysis System (SAS) (Freund et al. 1986).

The experiment was a three-level classic nested, unbalanced design. Therefore, the number of offspring in dams and in sires within sires should revise ("revise" means adjust). The Effective Means were computed using the equations:

Effective mean number of offspring in dams within sires:

$$K_1 = [N - \sum(n_{ij}^2/dn_i)]/(D - S)$$

Effective mean number of offspring in dams:

$$K_2 = [\sum(n_{ij}^2/dn_i) - \sum(n_{ij}^2/N)]/(S - 1)$$

Effective mean number of offspring in sires:

$$K_3 = (N - \sum dn_i^2/N)/(S - 1)$$

in which S = number of sires, D = number of dams, n_{ij} = number of offspring of the i -th sire and j -th dam, dn_i = number of offspring of i -th sire, and N = sum of number of offspring of all sires or all dams.

The phenotypic variance (V_p) was separated into the additive genetic variance (V_A), nonadditive genetic variance (V_N), and environmental variance (V_E), and the environmental variance (V_E) was separated into the common environmental variance (V_{EC}) and the specific environmental variance (V_{ES}) using the standard separation of variance components (Falconer 1989). The causal components of variance were estimated from the full- and half-sib covariance using the relationships in Table 2.

Heritabilities were computed using the relationships: $h^2 = V_A/[V_A + V_{NA} + V_E]$

*Corresponding author. E-mail: jhxiang@ms.qdio.ac.cn

TABLE 1.
Analysis of variance for components of phenotypic variation.

Source of Variance	Degrees of Freedom (df)	Sum of Squares (SS)	Mean Square (MS)	Expected Mean Square E(MS)
Dams	F × M - 1	SS _M		
Sires	F - 1	SS _F	MS _F	$\sigma^2 + k_2 \sigma_M^2 + k_3 \sigma_F^2$
Dams/sires	F × (M - 1)	SS _{M(F)}	MS _{M(F)}	$\sigma^2 + k_1 \sigma_M^2$
Offspring (error)	F × M × (n - 1)	SS	MS	σ^2
Total	N - 1	SS _T		

Thus, heritabilities in the narrow sense of paternal half-sib and maternal half-sib and full-sib were computed using the respective relationships:

$$h_{HS(S)}^2 = 4 \times \sigma_S^2 / (\sigma_S^2 + \sigma_D^2 + \sigma^2)$$

$$h_{HS(D)}^2 = 4 \times \sigma_D^2 / (\sigma_S^2 + \sigma_D^2 + \sigma^2)$$

$$h_{FS(D/S)}^2 = 2 \times (\sigma_S^2 + \sigma_D^2) / (\sigma_S^2 + \sigma_D^2 + \sigma^2)$$

Test of significant of heritability: $t = h^2 / \sigma_h^2$

Paternal half-sib:

$$V_{(h^2)} = \left(\frac{4}{\sigma_P^2} \right)^2 \left[V \left(\frac{MS_S}{k_3} \right) + V \left(\frac{MS_D}{K_3} \right) \right]$$

$$\sigma_h^2 = \frac{4}{\sigma_P^2} \sqrt{\frac{2}{k_3^2} \left(\frac{MS_S^2}{S-1} + \frac{MS_D^2}{N-D} \right)}$$

Maternal half-sib:

$$V_{(h^2)} = \left(\frac{4}{\sigma_P^2} \right)^2 \left[V \left(\frac{MS_D}{k_1} \right) + V \left(\frac{MS_E}{k_1} \right) \right]$$

$$\sigma_h^2 = \frac{4}{\sigma_P^2} \sqrt{\frac{2}{k_1^2} \left(\frac{MS_D^2}{D-S} + \frac{MS_E^2}{N-D} \right)}$$

Full-sib:

$$\sigma_h^2 = 2 \sqrt{\frac{2(1-r_{FS})^2[1+(k_1-1)r_{FS}]^2}{k_1(k_1-1)(D-1)}}$$

Experimental Animal

Collection and Maintenance

Parental *S. nudus* were taken from a cultured population in Dalian Bay on the northern coast of the Yellow Sea on 10 September 2001. These individuals were held at 18–22°C under 500 lx

illumination and fed *Laminaria japonica ad libitum* for 32 days before spawning on 12 October 2001.

Fertilization

Individuals were removed from the aquaria and allowed to drain for 30 min before 1 mL 0.5 M KCl was injected into the coelomic cavity via the peristomial membrane. They were placed on the tops of flasks filled with seawater, and the eggs and sperm were collected from each individual for 30–60 min.

The eggs of each female were fertilized with sperm from a single male. Approximately 150,000 eggs from each female were placed in 100-L containers. Sperm were diluted 1000-fold (Uehara et al. 1990), and a small amount of the diluted sperm was added to the eggs. Fertilization success was examined microscopically. The fertilized eggs were washed 2–3 times to remove excess sperm. The embryos were layered on the bottom of flasks and transferred into a 50-L container to develop at 17–21°C at a density of 4–5 individuals/mL. Normal plutei developed in 30–35 h (Rahman et al. 2000, Rahman et al. 2001).

Rearing

The larvae were transferred to 100-L containers of filtered seawater at 16°C. Densities of larvae from the 2-arm to the 8-arm stage were maintained at 1–2 individuals/mL. The water was changed twice daily. The larvae were fed *Chaetoceros gracilis*. Light was maintained at <300 lx. A small amount of air was bubbled into the water. After three months the juveniles from each fertilization group were placed separately into plastic cages suspended in a large pool. The juveniles were fed fresh *Laminaria japonica*. The cages were changed every 2 mo. The juveniles were weighed and their diameter measured at ages 3 and 5 mo.

RESULTS

Growth: Increase in Body Weight and Test Diameter

Mean and standard deviation of the increase in body weight and test diameter of offspring at 3 and 5 mo of age are given in Table 3.

TABLE 2.
Relationships between the covariance of full- and half-sibs and causal components of phenotypic variance.

Component of Variance	Covariance Components	Causal Components	Calculation of Component of Variance
σ_F^2	COV _{HS}	1/4 V _A	{MS _F - [(MS _{M(F)} - MS _E)/k ₁] × k ₂ - MS _E }/k ₃
σ_M^2	COV _{FS} - COV _{HS}	1/4V _A + 1/4V _{NA} + V _{EC}	(MS _{M(F)} - MS _E)/k ₁
σ_E^2	V _P - COV _{FS}	1/2V _A + 3/4V _D + V _{ES}	MS _E
$\sigma_T^2 = \sigma_F^2 + \sigma_M^2 + \sigma^2$	V _P	V _A + V _{NA} + V _{EC} + V _{ES}	
$\sigma_F^2 + \sigma_M^2$	COV _{FS}	1/2V _A + 1/4V _D + V _{EC}	

TABLE 3.

Body weight and test diameter of offspring at 3 and 5 mo of age.

Growth Phase	Body Weight (g)		Test Diameter (mm)	
	Average	Standard Deviation	Average	Standard Deviation
3 mo	0.014484	0.0103	2.916	0.945
5 mo	1.366	0.377	8.492	2.841

Analysis of Variance of Body Weight and Test Diameter of Offspring

Analysis of variance demonstrated great differences in body weight and diameter of juveniles from different females mated with the same male and between males at both 3 and 5 mo of age (Table 4).

Effective mean number of offspring for sires and dams after 3 mo was computed as follows: effective mean number of offspring in dams within sire is $K_1 = 34.689$, in dams is $K_2 = 38.821$, and in sires is $K_3 = 107.719$. Effective mean number of offspring for sires and dams after 5 mo was computed as follows: $K_1 = 33.505$, $K_2 = 36.831$, and $K_3 = 109.268$.

The causal components of variance were estimated from the full- and half-sib covariance using the relationships in Table 5.

Estimations of Heritability of Body Weight and Test Diameter of Offspring

Heritabilities in the narrow sense of paternal half-sib and maternal half-sib and full-sib of body weight and diameter of 3-mo-old *S. nudus* were calculated on the result of component of variance and test ($t = h^2/\sigma_e^2$) of significance of heritability, respectively, as shown in Table 6.

All of the heritability in the narrow sense of sire half-sib and the heritability in the broad sense of dam full-sib of body weight and test diameter of *S. intermedius* were significantly different from zero (t -test, $P < 0.01$).

Estimated heritability was somewhat different among the sire heritability, dam heritability, and the pooled (combined) heritabil-

ity for all three traits. The estimates of dam heritability were higher than those of sire heritability, and the pooled (combined) heritability was moderate.

DISCUSSION

Predicted heritabilities of larval growth have been reported for full-sib correlation analysis for *Crassostrea virginica* (Hadley et al. 1975, Lannan 1972, Newkirk et al. 1977) and *Penaeus vannamei* (Carr et al. 1997) and half-sib correlation analysis for *Mercentaria mercenaria* (Rawson and Hilbish 1990), *Macrobrachium rosenbergii* (Malecha et al. 1984), *Penaeus vannamei* (Benzie et al. 1996, Carr et al. 1997), and *Penaeus stylirostri* (Benzie et al. 1996). Predicted heritabilities of shell traits in wild *Littorina saxatilis* populations have been reported for full-sib correlation analysis and offspring-mother regression (Carballo et al. 2001). Estimates of heritabilities in the narrow sense generally ranged from 0.2 to 0.7. Realized heritability for increase in rate of growth in northern quahog and *Argopecten irradians concentricus* (Crenshaw et al. 1991, Crenshaw et al. 1996) and realized heritability estimates for growth in the Chilean oyster *Ostrea chilensis* (Toro et al. 1995). In our study, the estimates of heritabilities in the narrow sense for body weight at 3–5 mo of age ranged from 0.217 to 0.457, consistent with those reported for other species. However, estimates based on full-sib families bias heritabilities upwards when dominance and maternal effects are present (Lester 1988). Because of the nested design and a paternal half-sib correlation analysis used, the estimate reported here is more precise and unbiased. This is the first report of heritability in the narrow sense reported for sea urchins.

An animal model includes a random effect for the additive genetic effect of each individual; incorporates a complete set of additive genetic relationships among all individuals; and allows an unbiased estimation of variance components, even for the data involving selection and nonrandom mating (Gall and Bakar 2002, Sorensen and Kennedy 1986, Su et al. 1997). In the current investigation, full-sib family was taken as a random effect in a simple random model to account for the covariance among full-sibs caused by common environmental, dam, and nonadditive genetic

TABLE 4.

Analysis of variance for components of phenotypic variation of *Strongylocentrotus nudus* at 3 and 5 mo of age.

Source of Variance	Body Weight		Test Diameter			
	Degrees of Freedom (df)	Mean Square (MS)	F-Value	Mean Square (MS)	F-Value	Expected Mean Square E (MS)
3 mo						
Dam	62	4.08340×10^{-3}	9.893*	6.01406	6.737*	$\sigma^2 + k_2\sigma_M^2 + k_3\sigma_I^2$
Sire	20	7.26634×10^{-3}	17.605*	10.71717	12.006*	
Dams within sires	42	2.56772×10^{-3}	6.221*	3.77449	4.228*	$\sigma^2 + k_1\sigma_M^2$
Full-sibs within dams	2210	4.12753×10^{-4}		0.89266		σ^2
Total	2272					
5 mo						
Dams	59	0.79976	7.580*	69.16935	8.570*	$\sigma^2 + k_2\sigma_M^2 + k_3\sigma_I^2$
Sire	20	1.31728	12.485*	108.2978	13.418*	
Dams within sires	39	0.53436	5.065*	49.10348	6.084*	$\sigma^2 + k_1\sigma_M^2$
Full-sibs within dams	2045	0.10551		8.07108		σ^2
Total	2104					

* $P < 0.01$; S = sires, D = dams; k_1 is the weighed mean offspring number of females, k_2 is the weighed offspring number of females within sire, k_3 is the weighed mean offspring number of sires.

TABLE 5.

Relationships between the covariance of full- and half-sibs and causal components of phenotypic variance.

Component of Variance	Causal Components	Covariance Components	Result of Component of Variance			
			3 Mo		5 Mo	
			Body Weight	Test Diameter	Body Weight	Test Diameter
σ_F^2	$1/4 V_A$	Cov_{HS}	4.13895×10^{-5}	0.06027	0.00678	0.50447
σ_M^2	$1/4 V_A + 1/4 V_{NA} + V_{EC}$	$Cov_{FS} - Cov_{HS}$	6.16974×10^{-5}	0.08308	0.01280	1.22465
σ^2	$1/2 V_A + 3/4 V_D + V_{FW}$	$V_P - Cov_{FS}$	4.275×10^{-4}	0.89264	0.10551	8.07108
$\sigma_P^2 = \sigma_F^2 + \sigma_M^2 + \sigma^2$	$V_A + V_{NA} + V_{EC} + V_{EW}$	V_P	5.40587×10^{-4}	1.03598	0.12509	9.80020
$\sigma_F^2 + \sigma_M^2$	$1/2 V_A + 1/4 V_D + V_{EC}$	Cov_{FS}	1.03087×10^{-4}	0.14335	0.01958	1.72912

effects, and half-sib family was used to account for covariance among half-sibs caused by common environmental, dam, sire, and nonadditive genetic effects. The results from the analyses based on this model were expected to be unbiased estimates of genetic parameters for the base population.

The much larger heritabilities computed from the female additive genetic component indicate the female genetic component still contains common environmental effects, maternal effects, or non-additive genetic variance in the body weight or diameter. Dam effects are omnipresent in the study. The fact that the juvenile phase may indicate that the quality of yolk reserves plays a role in

early development. Dam effects may persist after the onset of exogenous feeding. Crandell and Gall (1993) reported that dam effects persist up to 2 y in rainbow trout and up to 18 mo in Arctic char (Nilsson, 1994).

The estimated heritability indicates significant additive genetic variation for body weight and test size over all the sampling periods. Sire heritability for the different variables were lower than dam heritability in many cases. However, all heritabilities in the narrow sense of body weight and test diameter of *S. intermedius* were significantly different from zero ($P < 0.01$). The heritabilities in the narrow sense estimate obtained from this experiment indicate there is sufficient variation in the base population of sea urchins to respond to natural or artificial selection on juvenile growth weight. This justifies selection of juveniles based on growth characteristics for cultured brood stock.

TABLE 6.

Heritabilities in narrow sense (h^2) and standard error (σ_{h^2}).

	3 Mo of Age		5 Mo of Age	
	Body Weight	Diameter	Body Weight	Diameter
h^2_S	$0.306^* \pm 0.055$	$0.233^* \pm 0.046$	$0.217^* \pm 0.042$	$0.206^* \pm 0.041$
h^2_D	$0.457^* \pm 0.071$	$0.321^* \pm 0.046$	$0.409^* \pm 0.064$	$0.500^* \pm 0.072$
h^2_{FS}	$0.381^* \pm 0.063$	$0.277^* \pm 0.051$	$0.313^* \pm 0.054$	$0.353^* \pm 0.058$

* Very significant between h^2 with zero ($P < 0.01$); $t_{(0.01)} = 2.576$.

ACKNOWLEDGMENTS

This is contribution number G1999012009 of 973 from the Chinese National Fundamental Research project and Chinese High Technology Plan (2002AA628170). We would like to thank J. Song, G. D. Wang, R. L. Xing, and S. G. Yan for their invaluable assistance in the laboratory production of the family lines. The authors thank J. M. Lawrence for editing the paper.

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UTILIZATION OF LOG-PIILING STRUCTURES AS ARTIFICIAL HABITATS FOR RED KING CRAB *PARALITHODES CAMTSCHATICUS*

BRADLEY G. STEVENS,* J. ERIC MUNK, AND PETER A. CUMMISKEY

National Marine Fisheries Service, Alaska Fisheries Science Center, Kodiak Fishery Research Center,
301 Research Ct., Kodiak, Arkansas

ABSTRACT Artificial habitats or reefs have been used to mitigate for alteration of marine habitats and increase populations of desirable marine species. In Kodiak, Alaska, breakwater construction covered 3.5 ha of sedimentary habitat potentially usable by commercially valuable red king crab *Paralithodes camtschaticus*. Juvenile king crab are common occupants of wooden dock pilings, suggesting that pilings could be used to mitigate for loss of natural habitat. To test this hypothesis, six log-piling structures were constructed from untreated spruce and placed in pairs at three different locations in ocean bays near Kodiak. Divers conducted a yearlong study of king crab recruitment by making quarterly counts of crab on the structures and adjacent seafloor areas. Abundance of juvenile (9–21 month-old) king crab increased steadily from June 1997 through March 1998 as crab recruited to the structures, then declined in June 1998. Crab abundance was significantly higher on piling structures than on the adjacent substratum. Site, season, and their interaction had significant effects on abundance. Why juvenile king crab are attracted to pilings is unknown. Pilings are not structurally complex habitats, but provide hard surfaces for fouling organisms such as hydroids, which are preferred habitat for juvenile crab. Additionally, pilings do not persist in the environment, and may not be the best structure for habitat enhancement. For these reasons, and because there is no evidence that red king crab are habitat-limited in our study area, we do not recommend the use of pilings as artificial habitats for red king crab.

KEY WORDS: king crab, habitat, ecology, settlement, artificial reefs, *Paralithodes*

INTRODUCTION

The use of artificial reefs for enhancement of marine populations has received considerable attention in recent years (for examples see Jensen 2002). Such structures may be assembled from a variety of components including natural (logs and rocks), semi-natural (concrete blocks, modules, or concreted boulders), and non-natural (tires, coal ash, wrecked ships, junked cars, and derelict oil platforms). Reefs have been constructed for a variety of recreational and commercial uses, including fisheries enhancement, scuba diving, aquaculture, habitat restoration, environmental mitigation, resource conservation, and research (Seaman 2002). Over time, research on artificial reefs has developed from purely observational studies of colonization, to studies of their ecological function, design and placement, and performance evaluation (Seaman 2002). A major issue in research on enhancement structures has been the “attraction-production” debate: do artificial structures support increased production of target species, or simply attract them from somewhere else? Conflicting viewpoints often depend on assumptions about whether reef-associated species are limited by habitat availability (Grossman et al. 1997) or recruitment variability, making artificial reefs little more than elegant fishing tools (Bohnsack et al. 1997). Lindberg (1997) argued that the answer depends on the specific structure, its location and objectives, and the species considered. Generally, production can be improved by use of highly complex structures that provide high levels of structural heterogeneity at appropriate scales. Such structures probably function to reduce mortality during critical life stages by reducing predation, and providing improved foraging opportunities (Bohnsack et al. 1997).

Most studies of artificial reefs to date have focused on highly mobile fauna such as reef-associated or pelagic fishes. Those dealing with benthic resources have addressed fouling organisms (Foster et al. 1994), or epibenthic prey species (Jara & Cespedes 1994)

of very low motility. Few have studied the use or impacts of artificial structures on commercially significant decapod crustaceans, which are highly motile within limited ranges. Notable exceptions include studies of Florida spiny lobsters *Panulirus argus* (Herrnkind & Butler 1986, Butler & Herrnkind 1997, Herrnkind et al. 1997a, Herrnkind et al. 1997b). These researchers have demonstrated that habitat availability limits the abundance of juvenile lobsters, and enhancement with appropriate artificial substrates can greatly increase abundance, whereas artificially increasing the abundance of juveniles by seeding is not effective. Such increases occur primarily through settlement rather than by immigration (Herrnkind et al. 1997a).

In 1997, a new breakwater was constructed by the United States Army Corps of Engineers (USACE) at the south end of St. Herman Harbor, Near Island, Kodiak, Alaska. During construction of the breakwater >3.5 ha of sedimentary habitat were covered with rock, at depths from 5–20 m, raising concerns that such habitat alterations might displace economically important marine species, particularly red king crab *Paralithodes camtschaticus* (Tilesius, 1815) (hereafter referred to as RKC).

At Kodiak, RKC up to 1.5 y of age are commonly found on wooden pilings covered with a variety of fauna as biologic structure, suggesting that pilings associated with piers are good habitat for juvenile crab. Based on the results of previous studies, (Dew et al. 1992) the National Marine Fisheries Service (NMFS) convinced the United States Coast Guard not to remove a condemned pier (Marginal Pier) in Womens Bay, Kodiak Island, Alaska, which contains hundreds of pilings and is a common site for juvenile king crab of 10–25 mm carapace length (CL). The USACE also installed 50 pilings at a site in Womens Bay as additional crab habitat; although these pilings were not routinely surveyed, we occasionally observed RKC there, though most of the pilings were destroyed by ice within a few years.

This study was undertaken to determine whether juvenile RKC would recruit to submerged log piling structures, and if they could be used to mitigate for habitat alterations caused by construction of breakwater and marina facilities.

*Corresponding author. E-mail: bradley.g.stevens@noaa.gov

MATERIALS AND METHODS

Six log piling structures (Fig. 1) were built and deployed in pairs. Each structure consisted of four 30-cm diameter corner posts (3 m spruce logs with intact bark) sunk into $0.6 \times 0.6 \times 0.5$ m concrete blocks. Adjacent and opposing pairs of corner posts were connected by horizontal 5×20 cm ($\leq 2 \times \leq 8$) beams of rough milled spruce, and similar beams connected the base of each post to the upper cross beam (see Fig. 1). Each structure had a surface area of approximately 35 m^2 . Two structures, labeled North (N) and South (S), were placed in approximately 10 m of water at each of 3 sites (Fig. 2): inside the breakwater (IB), outside the breakwater (OB), and in Womens Bay (WB), approximately 8 km away. The paired structures at the IB and OB sites were placed 27.4 m and 57.3 m apart, respectively. Although both sites were within 100 m of the rock breakwater, we did not survey it because the profusion of kelp prevented adequate sampling of the rock surface and crevices. In Womens Bay, the North and South structures were separated by about 600 m but were placed 34.1 m and 39.3 m, respectively, from Marginal Pier. Marginal Pier is a +60-year-old dock consisting of hundreds of creosote-treated wooden pilings, many of which have decayed, and most are covered with fauna including sponges, anemones, hydroids, bryozoans, barnacles, and sea stars. A fourth pair of survey sites included four Marginal Pier pilings nearest to each of the WBN and WBS habitats; these were labeled MPN and MPS, respectively. All log-piling structures were placed on the seafloor between May 19 and 22, 1997 prior to settlement of the 1997 year-class of RKC.

To compare counts of organisms on the structures to the surrounding environment, benthic transect lines were staked out on opposite sides of each structure. These also served as guidelines between the structures at the IB and OB sites. Because the IB structures were closest together, the standard transect length was defined as half the distance between them, or 13.7 m. Transects of similar length were established on the opposite sides of each structure as well. Surveys were conducted by scuba every 3 mo from June 1997 to June 1998 for a total of five quarterly samples.

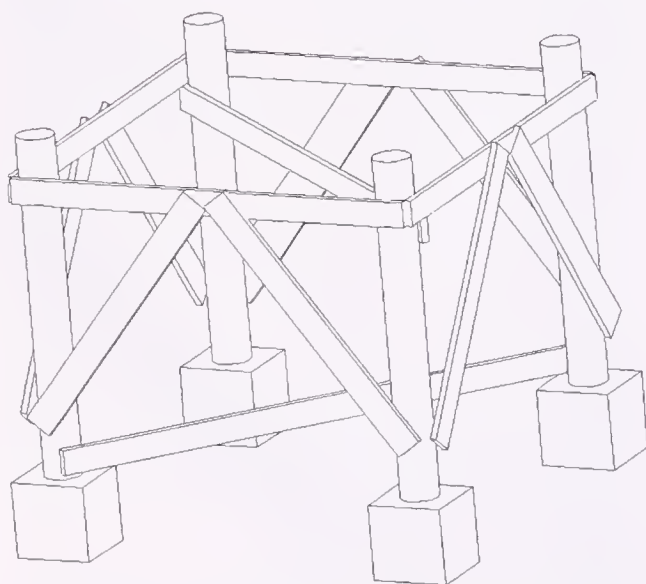


Figure 1. Design of the log piling structures used in the study. All were constructed of untreated, rough-milled spruce, held together by large bolts, and anchored by concrete bases.

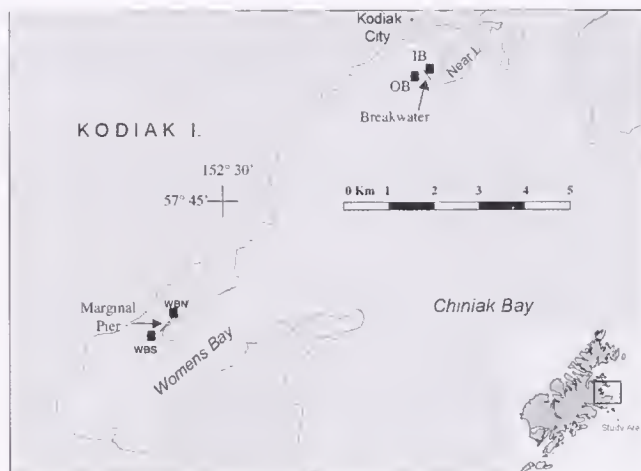


Figure 2. Location of Kodiak, Alaska, and sites of piling structure placement. IB, inside Breakwater; OB, outside Breakwater; WBN, Womens Bay North; WBS, Womens Bay South.

During each survey, divers descended a marker line to the bottom and counted all RKC on the outside and inside of the structure. Divers then counted RKC within a 1 m swath along both sides of the benthic transect lines. Each transect count was treated as an individual replicate. The total bottom area surveyed around each structure was 54.8 m^2 (2 transects \times 2 m \times 13.7 m). Divers then swam to the second structure at the site and repeated the structure and transect counts. Both structures at a site were surveyed on a single day, and each site on separate days within a week. At Marginal Pier, divers also counted RKC on the bottom 3 m of the four pilings nearest to each structure. The total data matrix consisted of 100 samples (5 seasons, 3 sites, 2 structures, 3 locations (structure, transect 1, transect 2), plus two MP samples in each season). To prevent disturbance or displacement, sizes of RKC observed by one diver (JEM) were estimated in 5-mm increments of carapace length (CL). During each dive, a series of replicate photographs were taken at specific locations, including piling tops, sides, crossbeams, concrete bases, and on the transects. Qualitative observations of substrate characteristics and sessile epibenthic species on the piling structures and transects were made from these photographs.

Preliminary data analysis showed that variances among samples were not homogeneous, indicating severe departures from normality that were not remedied by square root or angular trans-

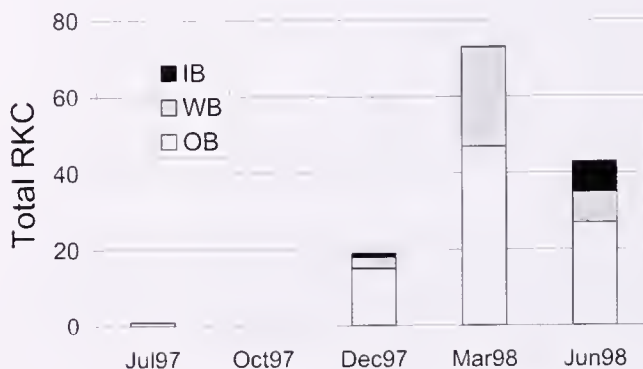


Figure 3. Counts of red king crab at each site, by sampling quarter. IB, inside Breakwater; OB, outside Breakwater; WB, Womens Bay.

formation. For this reason, and because the data are counts of (relatively) rare events, we chose to use a generalized linear model (GLM) type of ANOVA, based on a Poisson distribution rather than the normal distribution. The GLM allowed us to model RKC counts as a function of several factors. Only the data from the structures was analyzed in this manner; transect counts were excluded because of their low numbers, and counts from Marginal Pier were excluded because they were a different type of piling (older, creosote treated). The latter were only compared with the adjacent structures in Womens Bay. Factors included in the analysis were quarter, site, structure (north or south within pairs), and 2-way interactions between those 3 variables. Following this, a nonparametric Mann-Whitney *U*-test was used to make comparisons between pairs of sample sites. Sites compared were Marginal Pier versus site WB, site IB versus site OB, site IB versus WB, and OB versus WB. Statistical analysis was conducted using S-Plus version 4.5 or SPSS version 10.

RESULTS

A total of 136 RKC were observed and counted (Table 1). The largest number of RKC (90 crab, or 73% of the total excluding Marginal Pier) were observed at the OB site, followed by WB (25 or 20%), Marginal Pier (12), and IB (9). Excluding Marginal Pier samples, virtually all RKC (99%) were observed on the structures, and only two RKC were observed on the transects: one on kelp and one on a boulder. At each site, over twice as many RKC occurred on the southern structure (85) as on the northern (37). Only one RKC was observed (on the OB benthic transect) during the first 2 quarters (June and September 1997). By December 1997, young-of-the-year RKC <15 mm CL (9 mo post-hatch) were present on the structures, having probably settled there as post larvae (Table 2). Numbers increased through March 1998, but most were still <15 mm CL. A few crab >25 mm CL, representing the previous

TABLE 1.

Numbers of red king crab counted on log-piling structures, transects and Marginal Pier pilings, in four seasons, at three sites: IB (inside breakwater), OB (outside breakwater), and WB (Womens Bay).

Sample Period	Sub-stratum	IB	OB	WB	Total
June 1997	Structures	0	0	0	0
	Transects	0	1	0	1
	Marginal Pier			0	0
	Subtotal	0	1	0	1
Sept 1997	Structures	0	0	0	0
	Transects	0	0	0	0
	Marginal Pier			0	0
	Subtotal	0	0	0	0
Dec 1997	Structures	1	15	2	18
	Transects	0	0	0	0
	Marginal Pier			1	1
	Subtotal	1	15	3	19
March 1998	Structures	0	47	17	64
	Transects	0	0	0	0
	Marginal Pier			9	9
	Subtotal	0	47	26	73
June 1998	Structures	8	26	6	40
	Transects	0	1	0	1
	Marginal Pier			2	2
	Subtotal	8	27	8	43
Grand Total		9	90	37	136

TABLE 2.

Numbers of red king crabs in 5-mm categories of estimated carapace length (CL), observed on piling structures. Only one diver estimated sizes, so this table does not include all crabs observed.

CL Range (mm)	June 1997	Sept 1997	Dec 1997	March 1998	June 1998
10-14	1		3	20	1
15-19			1	4	1
20-24			1		28
25-29				1	
30-34					
35-39				1	1
40-44					1

yearclass (age +1, 21 mo post-hatch) were present on the structures, and probably arrived by immigration. Numbers of RKC observed on structures declined in June 1998; by this time, most crab were in the 20-24 mm CL range.

The Poisson-based GLM showed that the effects of quarter, site, and structure were all significant (Table 3). The interaction of quarter \times site was significant because the highest counts occurred in March 1998 at the OB and WB sites, but counts were highest in June 1998 at the IB site. The interactions of structure \times quarter and structure \times site were not significant. There were not enough degrees of freedom remaining to include a 3-way interaction, and it probably would not have been significant because no 2-way interactions involving structure were significant. The Mann-Whitney *U*-test showed no significant difference in counts of RKC between the WB and MP sites ($U = 45.0$, $P = 0.678$), WB and IB ($U = 39.5$, $P = 0.369$), or WB and OB ($U = 36.0$, $P = 0.267$). Differences between IB and OB were much greater, but still marginally non-significant ($U = 27.0$, $P = 0.057$).

Sediments inside the breakwater were silty and supported a dense community of tube-building polychaetes (possibly *Spiochaetopterus costarum*), whereas sediments outside the breakwater were sandier with scattered rocks and were devoid of epibenthos, except for occasional sea stars. Sediments near the WB structures were also silty, but scattered with mussel shells from Marginal Pier, and occasional anthropogenic debris from 50 y of military use. Calcareous tube-building polychaetes (probably *Serpula vermicularis*) and plumose anemones (*Metridium senile*) were abundant on Marginal Pier and adjacent debris.

TABLE 3.

GLM analysis for effects of factors on counts of red king crabs on piling structures (N or S) in five quarters, at three sites. Data from transect counts and marginal pier are excluded. All data were transformed to log ($x + 1$) prior to analysis.

Source	df	Res. Dev.	P
Null	29	293.24	<0.001
Quarter (QTR)	25	141.21	<0.001
Site (SIT)	23	56.83	<0.001
Structure (STR)	22	33.94	<0.001
QTR \times SIT	14	15.31	0.017
QTR \times STR	10	12.15	0.532
SIT \times STR	8	7.93	0.121

By July 1997, approximately 3 wk after placement of the structures, barnacles of 1–2 mm diameter had colonized most of the structures. Mottled sea stars (*Evasterias troschellii*), sunflower stars (*Pycnopodia helianthoides*), green sea urchins (*Strongylocentrotus droebachiensis*) and sculpins (family Cottidae) arrived on the structures via immigration. By September 1997, small clumps of green algae and hydroids were growing on most of the structures, and filamentous red algae were common on the WB structures. Densities of epibenthos were lower inside the breakwater than outside. By December, the structures had been colonized by encrusting bryozoans, decorator crab (*Oregonia gracilis*), RKC, calcareous tubeworms, and nudibranchs (*Flabellina fusca*). In March, red algae (*Palmaria* sp.), *Laminaria* sp., tunicates (probably *Molgula* sp.), and hydroids were abundant. Filamentous red algae obscured the sheltered sediments inside the breakwater, but were absent outside the breakwater. By June, piling tops were once again covered with green algae, and helmet crab (*Telmessus cheiragonus*), hermit crab (*Pagurus* sp.), and RKC were common on the structures.

DISCUSSION

Our data demonstrate that log piling structures may serve as potential habitats for juvenile RKC. Numbers of crab on the structures were two orders of magnitude greater than on the adjacent seafloor. As a result of this work, we recommended to the USACE that new dock structures in the Kodiak area be built with pilings rather than sheet-metal bulkheads with backfill, as proposed by some developers. Use of pilings preserves the underlying seafloor substratum that would be covered by fill, and adds hard vertical structure useful to crab and other fauna. We cannot recommend that piling structures be deployed to mitigate for habitat loss at this time for several reasons. First, the reason why juvenile RKC are attracted to pilings is still not understood, though settlement among fouling organisms seems most likely. Second, pilings have limited surface area and low fractal dimension; that is, they are devoid of highly complex interstitial spaces, which is apparently the structural feature that makes various natural and artificial habitats attractive to juvenile RKC (Stevens & Kittaka 1998 and Stevens 2003). The relationship between the scale of refuge spaces and the range of body sizes is an important factor in survival of reef-associated decapods (Eggleston et al. 1992). If habitat enhancement is deemed worthwhile for future research, then other types of structures might be more effective by providing a greater variety of interstices in a more compact structure. Furthermore, wooden pilings do not last long in a marine environment unless treated with toxic chemicals, and steel or concrete pilings may not attract the same fouling community. Moreover, in this study we evaluated only one kind of structure; better structures might include crushed rock or gravel, or specifically designed man-made substrata. It is possible that the new breakwater provides more habitat of better quality than the original sedimentary substratum that it replaced.

In our study, RKC were more abundant at a site (OB) that was exposed to prevailing weather and current, and less abundant at a nearby site (IB) protected behind a large breakwater, or at the head of Womens Bay, several kilometers from open water. This distribution could result from recruitment processes involving the transport of larvae by ocean currents until they reach suitable exposed habitat sites. As more larvae settle on exposed sites, fewer would remain in the water to settle at protected sites. The breakwater and its many crevices may also have "filtered" out many larvae prior to

their arrival at the IB site. This hypothesis could also account for the higher numbers of RKC on the southernmost habitats within each pair. At each site, exposure to the open ocean decreased, and distance from it increased, along an axis from south to north. The abundance of infaunal polychaete tubes at the IB site is indicative of sheltered habitat, whereas lack of their presence at the OB site was probably the result of wave action and current scour. RKC probably arrived on the structures by settlement as postlarvae during the late summer and fall of 1997, but were too small to be seen by divers until December. Observed numbers of RKC increased through March 1998, then declined the following summer. By that time, the earliest arrivals were 15 mo old and 15–20 mm CL. At that size, they are less vulnerable to predation, so may have emigrated from the structures. Newer recruits either did not replace them at the same rate, or were not yet large enough to be seen by divers. One of the most important species of the epibenthic community are hydroids because they are important habitat for newly settled RKC glaucothoe (postlarvae), which choose them over alternative habitats due to their complex 3-dimensional structure (Stevens 2003). Hydroids were abundant among artificial collectors made of onion sacs stuffed with monofilament line (Donaldson et al. 1992 and Blau & Byersdorfer 1994), and may be one reason that glaucothoe settle there en masse. Hydroid colonies do not develop until late summer, and their presence on the pilings and buoy lines may attract settling RKC glaucothoe to the pilings. However, hydroids were not highly abundant when the first recruits began to appear, and most crab were observed on bare wood.

Biologic structures are important settlement habitats for juvenile RKC and are practically the only location where they are found in many parts of their geographical range, such as the Bering Sea, where physical structure is scarce. This distribution is the result of habitat selection by settling postlarvae (glaucothoe) rather than of predation. During their first year of life, juvenile RKC are associated with sponge and bryozoan colonies (Sundberg & Clausen 1977), mussel and hydroid colonies (see photo in Stevens 2003), stalked ascidians and polychaete tubes (Stevens & MacIntosh 1991), and shell debris and cobble (McMurray et al. 1986 and Loher & Armstrong 2000). Glaucothoe will settle in large numbers on various types of artificial collectors (Donaldson et al. 1991 and Blau & Byersdorfer 1994). In the laboratory RKC glaucothoe prefer structurally complex habitats, whether artificial (aquarium filter material) or biologic (hydroids and complex red algae), to those with less structure (gravel) and will not settle on structure-less open sand (Stevens & Kittaka 1998 and Stevens 2003). Selection for such habitats is probably an adaptive response to high predation levels. At sizes of 10–15 mm CL, RKC are often observed "hitch-hiking" on sea stars (Dew 1990). At sizes >25 mm CL, RKC start to exhibit aggregative (podding) behavior (Powell & Nickerson 1965 and Dew 1990).

Crab and lobsters are attracted to, and use, various artificial habitats. In Chile, *Cancer edwardsi* and *Homalaspis plana* use the hollow spaces in artificial reefs made of concrete blocks (Jara & Cespedes 1994). Numbers of both species increased significantly on the reef and in quadrat samples taken from the surrounding substratum, in contrast to our data, which showed virtually no RKC present on surrounding substrata. Adult stone crab *Menippe* sp. were more abundant among concrete block reefs spaced at 60 m intervals than among those spaced at 2 m intervals, and abundance was strongly seasonal (Frazer & Lindberg 1994). Stone crab apparently arrived by immigration as adults and foraged among the surrounding substratum, in contrast to RKC that settle on pilings as

postlarvae, and were rarely observed "off structure". Reefs made of cemented coal-ash were used by spider crab *Maja squinado*, velvet swimming crab *Liocarcinus puber*, and European lobster *Homarus gammarus*, the latter of which were predominantly adults, indicating that recruitment occurred through immigration rather than by settlement (Jensen et al. 1994). Caribbean spiny lobster *Panulirus argus* are attracted to "casitas" consisting of concrete blocks (Lozano-Alvarez et al. 1994), or concrete over a PVC frame (Eggleston et al. 1992). Despite the presence of crab and octopus, artificial shelters may reduce predation on small juvenile lobsters, and thus increase production of that sizegroup (Eggleston et al. 1992). In Delaware Bay, the biomass associated with concrete artificial reefs was 2 to 3 orders of magnitude greater than that of the surrounding substratum, although no large decapods were observed there (Foster et al. 1994 and Steimle et al. 2002).

Population enhancement is probably feasible for commercially valuable decapods like spiny and American lobster because their abundance is limited by the availability of their preferred habitats. Postlarval spiny lobsters settle preferentially in clumps of red algae (*Laurencia* sp.) (Herrnkind & Butler 1986). Enhancement of natural habitat (algal clumps) or addition of artificial shelters (concrete blocks) is an effective way to increase the abundance of juvenile lobster, whereas seeding with postlarvae or juveniles is not effective

(Butler & Herrnkind 1997 and Herrnkind et al. 1997a). Enhancement of natural substrata with gravel plots caused densities of postlarval American lobsters (*Homarus americanus*) to increase by a factor of 10, whereas supplemental postlarvae declined by a factor of 5 (Wahle 1991). At present, there is no convincing evidence that RKC populations are limited by lack of available settlement habitat, so population enhancement by the addition of artificial habitats may not be effective for this species. Further research on settlement behavior of postlarval RKC, and shelter use by juveniles, both in the laboratory and in their natural environment, is warranted before enhancement of habitat or populations is undertaken with this species.

ACKNOWLEDGMENTS

This project was partially funded by the United States Army Corps of Engineers under MIPR Nos. E86954046-0001 and F12961362-0001. The piling structures were built and installed by Majdic and Sons, Inc. The authors thank W. E. Donaldson for assistance with diving surveys, I. Vining for help with statistical analysis and interpretation, and C. Armistead for production of maps. The manuscript benefited greatly from reviews by R. S. Otto, A. Stoner, and R. Stone.

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BIOLOGICAL CONTROL OF AQUARIUM PEST ANEMONE *AIPTASIA PALLIDA* VERRILL BY PEPPERMINT SHRIMP *LYSMATA* RISSO

ANDREW L. RHYNE*, JUNDA LIN, AND KATHIE J. DEAL**

Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida 32901

ABSTRACT Caridean shrimp in the genus *Lysmata* Risso are widely traded in the aquarium hobby industry. Peppermint shrimp *Lysmata* spp. have been used to control the brown (glass) anemone, *Aiptasia pallida* Verrill, a significant pest in the aquarium environment. A study was conducted to quantify the ability of two peppermint shrimp in controlling *A. pallida*. *Lysmata* sp. (Haiti variety) tended to have a higher consumption rate of sea anemone than *Lysmata* sp. (Florida Gulf Coast variety). Shrimp in a group tended to have a lower consumption rate per shrimp than individual shrimp in both species, but were capable of preying upon larger anemones. Larger (euhermaphrodite-phase) *Lysmata* (Florida Gulf Coast variety) had a higher rate of sea anemone consumption than that of smaller (male-phase) shrimp.

KEY WORDS: *Aiptasia pallida*, anemone, *Lysmata*, peppermint shrimp, predation

INTRODUCTION

Species of caridean shrimp *Lysmata* are commonly traded in the marine aquarium industry due to their striking coloration, beneficial symbiotic relationships, and quick acclimation to a captive environment. Many *Lysmata* species also feed on parasites of fish (Van Tassel et al. 1994; but see Spotte 1998), and therefore play an important role in the natural and aquarium environments.

The cleaner shrimps *L. amboinensis* De Mann, *L. grabhami* Gordon, and *L. debelius* Bruce are revered for their ability to clean fish and their brilliant coloration, therefore command a high market price. Peppermint shrimp, several species of *Lysmata* with red or maroon stripes along the entire length of their translucent bodies, are not as brightly colored. However, they are in high demand for their apparent ability to control the common marine aquarium pest anemone *Aiptasia pallida*. Though the unit price is much less than that of *L. amboinensis*, *L. grabhami*, and *L. debelius*, the total volume sold annually greatly exceeds that of any other shrimp in the marine aquarium industry. Although peppermint shrimp are marketed under the name *L. wurdemanni* Gibbs, the vast majority of shrimp sold are in fact a novel species misidentified as *L. ratliffi* Chace (Rhyne 2002, Rhyne & Lin, in preparation). There are clear morphologic and some distribution differences, evidences of reproductive incompatibility and species-level genetic differences in the group. This confusion has resulted in unreliable reports of the most effective species for controlling *A. pallida*. Another unique feature of *Lysmata* species is their reproductive system, protandric simultaneous hermaphroditism (Bauer 2000), in which shrimp first mature as males and then change to simultaneous hermaphrodites. A proportion of the shrimp, influenced by group size, may remain as males (Lin & Zhang 2001).

Brown (glass) anemone *Aiptasia pallida* (Aiptasiidae) is a common pest in marine aquariums. They are commonly found in shallow and nutrient-rich waters along the subtropical and tropical Atlantic coasts. The brown color is caused by a unicellular symbiotic zooxanthae, *Symbiodinium microadriaticum* Freudenthal, which allows the anemone to thrive in highly illuminated environments (Trench 1993), including the marine reef aquariums. *Aiptasia* rapidly reproduce asexually by pedal laceration (budding from their pedal disc) (Hunter 1984). When it occurs in dark places, *A.*

pallida is not brown due to loss of zooxanthae, but rather a pure palloidal white, hence the second half of its Latin name.

Stinging nematocysts within the anemone tentacles contain the venom beta phospholipase A2 and are used to stun prey or defend against potential predators (Grotendorst & Hessinger 2000). In the aquarium, these anemones harm both soft and hard corals and other sessile invertebrates, causing irritation and eventual death. The transparency and cryptic nature of *Aiptasia* results in its regular introduction into the marine aquarium from live rock or corals. *Aiptasia* cannot be eliminated by regular cleaning of aquarium. Many aquarium owners attempt to crush the anemones, which often promotes their proliferation and colonization in other areas of the tank. *Aiptasia* is commonly eliminated chemically and biologically. Chemical elimination is effective, but requires the laborious introduction of toxic injections directly into the oral disc of each anemone. Biologic control via anemone's natural predators eliminates the use of toxins. The nudibranch *Berghia verrucicornis* Costa (e.g., Carroll & Kempf 1990), butterflyfish (*Chelmon rostratus* Linnaeus and *Chaetodon kleinii* Bloch), and peppermint shrimp are commonly used as successful natural predators of *Aiptasia*. However, there is no scientific study to quantify the effectiveness of such control.

The purpose of this study is to document the predation of two peppermint shrimp species, *Lysmata* sp. (Haiti variety) and *Lysmata* sp. (Florida Gulf Coast variety) on *A. pallida*. Though both shrimp are novel species, they are both commonly traded as *L. wurdemanni* Gibbs.

MATERIALS AND METHODS

The experiments were conducted at the Aquaculture Laboratory, Florida Institute of Technology, in 2002. *Aiptasia pallida* specimens were obtained from a local aquarium store and cultured in a 40-L tank. Anemones were fed live *Artemia* nauplii under constant light for 60 days and allowed to asexually reproduce on the glass walls of the aquarium. A razor blade was used to gently scrape the pedal disc base of the anemones, and the anemones were then placed on the rough surfaces of ceramic tiles. Each tile (11 × 11 cm) was carefully placed in a 10-L aquarium, and anemones were allowed to adhere to the tiles for a minimum of 24 h. Location of anemones on the tiles was haphazard due to the natural movement of anemones during reattachment.

Two species of peppermint shrimp, *Lysmata* sp. (Florida Gulf Coast variety) (collected from north of Tampa Bay) and *Lysmata*

*Corresponding author. E-mail: arhyne@fit.edu

**Present address: Department of Marine and Environmental Systems, Florida Institute of Technology, Melbourne, Florida 32901.

sp. (Haiti variety, collected from Haiti) were tested as anemone predators. The Florida Gulf Coast variety used in the current study was referred to as "*L. wurdemanni*" in some past studies (Zhang et al. 1998a, Zhang et al. 1998b). These shrimp do not conform to any current species definition, but are popularly (and mistakenly) referred to as *L. rathbunae* (see Debelius 2001 for photographs). We will refer to this species as *Lysmata* (gulf) in this paper. The Haiti variety was incorrectly recorded as *L. wurdemanni* by Holthuis (1959) and was referred to as such by Lin and Zhang (2001), and will be referred to as *Lysmata* (Haiti). This shrimp has pink gonads and a carmine red transverse stripe on the third abdominal segment, whereas *Lysmata* (gulf) has green gonads, a dark crimson telson, and does not have a transverse pigment stripe on the third abdominal segment. Both novel species tested are currently being described as part of a review of the peppermint complex found in the Western Atlantic (Rhyne and Lin, in preparation).

Test shrimp were maintained in a recirculating system. Each shrimp was placed into a marked container and fed frozen adult *Artemia* once daily in the afternoon after anemone feeding trials. Salinity and temperature were maintained at 35 and 26 °C, respectively, for all the experiments.

To compare the consumption rates of the sea anemones (5–10 mm in oral disc diameter) by the two shrimp species, nine replicate shrimp of *Lysmata* (gulf) and *Lysmata* (Haiti), respectively, that had not previously fed on *Aiptasia* were used. Total length (TL) of each shrimp was measured. A single shrimp was placed into an enclosure within a 10-L aquarium that contained a tile with 9 to 11 anemones and allowed to acclimate for 15 min before being released into the aquarium. The time between release and each feeding and total number of anemones consumed were recorded. The shrimp was observed until all the anemones were eaten or for 24 h. An additional experiment was conducted to examine the feeding rate and behavior if multiple shrimp were present in an aquarium. Four groups (two for each species) of five shrimp of similar sizes were used. Each group was placed in a 10-L aquarium with a tile of 10 anemones. Again, the shrimp were confined to a holding pen and allowed to acclimate for 15 min prior to being released. The time from the shrimp's release to each feeding was recorded. The shrimp was allowed to feed until all the anemones were consumed.

To test the effect of shrimp size on its ability to feed on sea anemones, six large euhemaphrodite-phase and eight small male-phase *Lysmata* (gulf) were introduced individually into a 10-L aquarium with sea anemones (5–10 mm oral disc diameter) attached on a single tile. There was an average (\pm SD) of 14.8 (\pm 2.2) and 9.6 (\pm 3.1) anemones for each large and small shrimp, respectively. Each shrimp was confined to a holding pen and allowed to acclimate to the test aquarium conditions for 15 min prior to release. The number of sea anemone consumed was recorded after 24 h.

RESULTS

The average (SD) TL of *Lysmata* (gulf) and *Lysmata* (Haiti) was 24.1 (4.0) and 23.3 (5.2) mm, respectively. One shrimp of each species did not consume any anemone in 24 h. Excluding these two nonfeeding shrimp, an average (SD) of 9.1 (0.8) anemones was consumed by *Lysmata* (Haiti) in an average (SD) of 73.1 (26.8) min; and an average (SD) of 6.1 (2.4) anemones was consumed by *Lysmata* (gulf) in an average (SD) of 129.3 (78.9) min. The average (\pm SD) consumption rate of *Lysmata* (Haiti) (10.1 ± 9.5 per h) is not significantly higher than that of *Lysmata* (gulf) (6.8 ± 12.1 per h), due to large variations. The large variation in each species is due to one "outlier." If the "outlier" is removed

from each species, the average (\pm SD) consumption rate of *Lysmata* (Haiti) (6.8 ± 1.6) is significantly (*t* test, $P < 0.05$) higher than that of *Lysmata* (gulf) (2.5 ± 1.3). *Lysmata* (gulf) also had a significantly (*t* test, $P < 0.05$) longer average (\pm SD) response time (10.9 ± 10.4 min) (from release to first feeding) than that of *Lysmata* (Haiti) (2.3 ± 3.7 min).

The average (SD) TL of the first group of *Lysmata* (Haiti) was 18.0 (2.1) mm and it took the shrimp 48 min to consume the 10 anemones. The average (SD) TL of the second group of *Lysmata* (Haiti) was 19.0 (2.2) mm, and it took the shrimp 78 min to finish the 10 anemones. For *Lysmata* (gulf), the average (SD) TL of the first group was 22.0 (2.1) mm, and it took the shrimp 90 min to consume the 10 anemones. The average (SD) TL of the second group was 20.5 (4.1) mm, and it took the shrimp 31 min to consume the 10 anemones.

Large individuals (average \pm SD, TL = 41.0 ± 3.0 mm) of *Lysmata* (gulf) consumed an average (SD) of 12.8 (4.3) anemones [out of 14.8 (2.2) offered] in 24 h, significantly (*t* test, $P < 0.05$) higher than that consumed [5.4 (4.3) anemones out of 9.6 (3.1) offered] by the small shrimp (average \pm SD, TL = 19.9 ± 1.6 mm) during the same time period.

DISCUSSION

Both *Lysmata* species are capable of controlling *Aiptasia pallida*. *Lysmata* (Haiti) tended to have a higher predation rate and shorter response time than *Lysmata* (gulf). In *Lysmata* (gulf) at least, larger (euhemaphrodite-phase) shrimp consumed more anemones than smaller (male-phase) shrimp. Shrimp in a group tended to have a lower consumption rate per shrimp than individual shrimp. This may be a result of social interactions among the shrimp. Group feeding was not observed as cooperative, but competitive. In most cases, only one shrimp in a group was observed feeding on a small anemone at a time. However, a group of shrimp would feed on large anemones together and were able to consume larger anemones than an individual shrimp (unpublished data). Group feeding appeared to be cooperative for feeding on large anemones and competitive for feeding on small ones.

When attacking anemones, shrimp were prone to be stung with nematocysts and usually chose smaller, less harmful anemones. Peppermint shrimp appear to locate randomly the first anemone. After consuming the first anemone, the shrimp were observed readily seeking out other prey. The consumption of the first anemone appeared to invoke a feeding response. A shrimp would normally repeatedly approach the anemone until the anemone withdrew its tentacles. Then the shrimp would begin to consume voraciously the anemone. Some shrimp were observed making predation attempts, but were never successful. These shrimp may have molted recently, and their exoskeleton may be more sensitive to nematocysts (personal observation).

We observed that even satiated shrimp (after being fed with *Artemia* nauplii for an hour) would attempt to attack the anemones, but rescinded after being stung by the nematocysts. After a period of 24 h, the shrimp would successfully prey on anemones. While this study does not address the ability of shrimp to eat anemones over 10 mm oral disc diameter, both large shrimp (>3 cm TL) and groups of three to five shrimp have been observed to consume large (20 mm oral disc diameter) anemones (unpublished data).

ACKNOWLEDGMENTS

The authors thank Brandi Sloss and Vanessa Maxwell for assistance in the laboratory.

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EFFECTS OF SALINITY AND DIETARY CARBOHYDRATE LEVELS ON GROWTH AND ENERGY BUDGET OF JUVENILE *LITOPENAEUS VANNAMEI*

XINGQIANG WANG, SHEN MA,* SHUANGLIN DONG AND MEI CAO

Mariculture Research Laboratory, Fisheries College, Ocean University of China, Qingdao, 266003, People's Republic of China

ABSTRACT The culture of *Litopenaeus vannamei* in low-salinity waters is now popular in many regions of the world. A 6×3 factorial experiment was conducted to determine the effects of salinities (tap water, 1‰, 2‰, 4‰, 8‰, and 16‰) and dietary carbohydrate levels (15.47%, 29.15%, and 41.00%) on survival, growth, food consumption, food efficiency, absorption efficiency, and energy budget of juvenile *L. vannamei*. The results showed that no shrimp survived in tap water at the end of the experiment irrespective of dietary carbohydrate (CBH). At each dietary CBH level, the specific growth rate (SGR), food consumption, and food efficiency generally increased with increasing salinity within the range of 1–16‰. At salinities of 1‰, 2‰, 4‰, 8‰, and 16‰, optimal CBH levels corresponding to maximum SGR decreased with increasing salinity and were 29.87%, 27.59%, 26.85%, 26.25%, and 22.29%, respectively. At 1–8‰, the percentage of consumed energy deposited in growth (%C) was significantly higher in shrimps fed 29.15% CBH as compared with other treatments, whereas at 16‰, the significantly higher value was in those fed 15.47% CBH.

KEY WORDS: *Litopenaeus vannamei*, salinity, carbohydrate, growth, energy budget

INTRODUCTION

The shrimp-farming industry is currently experiencing major crop losses due to disease outbreaks sometimes associated with the deterioration of water quality, which makes the establishment of saltwater ponds economically risky. Instead, the culture of penaeid shrimp in low-salinity waters, which not only provides a cost-effective solution to prevalent disease problems but also improves the use of saline-alkaline land waters, is now popular in many regions of the world (Bray et al. 1994; Dong 2003). The Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) possesses the ability to tolerate low salinities. Recently, there has been increasing interest in the culture of this species in low-salinity waters or fresh water (Saoud et al. 2003), for which information is required on the effects of salinity on performance, principally growth and survival. A reduction in salinity may modify the osmotic and ionic balances in shrimp. To recover the normal balances, the shrimp may have to expend a considerable amount of energy, resulting in less energy to spare for growth (Bindu & Diwan 2002; Zhang & Dong 2002).

In general, glucose is one of the main energetic substrates for general metabolic processes in crustaceans (Hu 1958). In a recent study, Rosas et al. (2000) reported that *Litopenaeus stylirostris* can use the gluconeogenesis pathway according to dietary carbohydrate availability, with a higher gluconeogenic activity in shrimps fed diets containing comparatively low amounts of carbohydrate and low or no gluconeogenic activity in shrimps fed diets containing more than 20% carbohydrate. This better efficiency of using carbohydrate for routine metabolism, thus liberating protein for tissue aggregation, was also reported in *L. vannamei* (Dokken 1987), *Penaeus monodon* (Alava & Pascual 1987), and *Penaeus indicus* (Ali 1982).

Taking into account the energy consumption required for osmoregulation of *L. vannamei* adapting to low salinity conditions (Bindu & Diwan 2002), it is possible that carbohydrate metabolism adaptation takes place. This study was designed to determine the effects of tap water (about 0.2‰ salinity) and low salinities (1‰, 2‰, 4‰, 8‰, and 16‰) on growth of juvenile *L. vannamei* fed

three levels of dietary carbohydrates (15.47%, 29.15%, and 41.00%), together with an analysis of the bioenergetic mechanisms involved.

MATERIALS AND METHODS

Diet Preparation

Three approximately isoenergetic diets were formulated, containing 15.47%, 29.15%, and 41.00% of carbohydrate. The diets were prepared by mixing dry ingredients and water (2:1, w/w), and pellet-type diets were produced through a meat grinder with a 1.6 mm die. The diets were then oven-dried (70°C for 12 h) to 6–7% moisture and stored at 4°C until used. The formulation and proximate composition of the experimental diets are shown in Table 1.

Source and Acclimation of Experimental Shrimp

The experiment was carried out at the Laboratory of Aquaculture Ecology, Ocean University of China, Qingdao, P.R. China. *L. vannamei* juveniles were obtained from Hainan Shrimp Breeding Farm, Hainan, P.R. China. The shrimp were initially held in six fiberglass tanks (200 × 80 × 60 cm) at 31‰ for three days and were acclimated to six salinity levels (16‰, 8‰, 4‰, 2‰, 1‰, and tap water) by lowering the salinity at a rate of 5‰ d⁻¹ by adding tap water (about 0.2‰ salinity).

Experimental Design

A two-factor factorial experimental design (6 × 3) with three replications per treatment was followed. A static-water system consisting of 54 aquaria (45 × 30 × 30 cm, water volume 35 L) with aeration was used. After 24 h feed deprivation, 48 shrimp with weight range from 0.625 g to 0.645 g were selected from the acclimating fiberglass tanks. From the 48 shrimp, three groups of four shrimps each were randomly sampled for analysis of initial energy content and nitrogen content at the corresponding salinity level. The remainder (36 shrimp) were randomly stocked into nine aquaria with each aquarium holding four shrimps. The experiment lasted for five weeks. Shrimp were hand-fed an excess ration twice daily (at 06:00 and 18:00 h) for each group. Each meal lasted approximately 2.5 h, and any uneaten food was collected and dried at 70°C. Twice a day, intact feces and molts were collected from

*Corresponding author. E-mail: mashen@mail.ouc.edu.cn

TABLE 1.

Ingredient and proximate composition of the experimental diets.

Formulation (%)	Carbohydrate Level (%)		
	15.47	29.15	41.00
Fish meal	47.5	31	28
Soybean meal	20	20	0
Peanut meal	20	10	0
Wheat flour	0	25	58
Fish oil	3	4.5	4.5
Lecithin	2	2	2
Sodium alginate	1.5	1.5	1.5
Vitamin mix ¹	2	2	2
Mineral mix ²	4	4	4
Proximate analysis (%)			
Moisture	6.51	6.65	6.71
Protein	45.31	35.18	26.59
Lipid	7.08	7.03	7.07
Carbohydrate	15.47	29.15	41.00
Ash	13.56	10.67	9.35
Gross energy (kJ · g ⁻¹)	17.77	17.54	17.38

¹ Vitamin mix, each 1000 g of diet contained: thiamin-HCl, 60 mg; riboflavin, 100 mg; folic acid, 10 mg; pyridoxine-HCl, 140 mg; niacin, 400 mg; calcium pantothenate, 140 mg; choline chloride, 4000 mg; inositol, 4000 mg; ascorbic acid, 4000 mg; biotin, 2 mg; p-amino benzoic acid, 150 mg; α-tocopherol, 400 mg; menadione, 34 mg; cyanocobalamine, 0.8 mg; retinol acetate, 150,000 IU; cholecalciferol, 60,000 IU.

² Mineral mix, each 1000 g of diet contained: Ca(H₂PO₄)₂, 8.800 g; CaCO₃, 8.240 g; K₂HPO₄, 4.000 g; NaH₂PO₄, 11.200 g; MgSO₄ · 7H₂O, 5.095 g; KCl, 1.600 g; FeSO₄ · 7H₂O, 0.400 g; AlCl₃ · 6H₂O, 0.016 g; ZnSO₄ · 7H₂O, 0.432 g; MnSO₄ · H₂O, 0.080 g; CuSO₄ · 5H₂O, 0.008 g; CoCl₂ · 6H₂O, 0.112 g; KI, 0.016 g; Na₂SeO₃, 0.001 g.

each aquarium, freeze-dried immediately, and held at 20°C until analyzed. For each aquarium, two-thirds of the water was replaced daily. During the experiment, dissolved oxygen was maintained above 6.0 mg/L, water temperature at 25 ± 0.5°C, pH from 7.7 to 8.2, and the photoperiod was 14L:10D.

Calculation of Data

Specific growth rate (SGR), food efficiency (FE_f), energy conversion efficiency (FE_e), and absorption efficiency (AE) were calculated as follows:

$$\text{SGR} = 100 \times (\ln W_2 - \ln W_1) / t$$

$$\text{FE}_f = 100 \times (W_2 - W_1) / C_w$$

$$\text{FE}_e = 100 \times (E_2 - E_1) / C_e$$

$$\text{AE} = 100 \times (C_w - F_d) / C_w$$

where W_2 and W_1 are the final and initial wet weights of the shrimp, E_2 and E_1 are the final and initial energy contents of the shrimp, C_w is the food consumption in dry weight, C_e is the consumed energy, F_d is the fecal production in dry weight, and t is the duration of the experiment.

The energy budget equation of *L. vannamei* juveniles can be defined through the construction of a budget in which the consumed energy (C) is partitioned between growth (G), respiration (R), excretion (U), feces (F), and exuviae (E), i.e., $C = G + R + U + F + E$. The gross energy contents of diet, shrimp, feces, and molt were measured with a 1281 Oxygen Bomb Calorimeter (Parr Instrument, Moline, Illinois). The initial energy contents of the

shrimp were assumed to be equal to the average energy value of the shrimp sacrificed at the beginning of the experiment.

Preliminary experiment indicated that nitrogen in *L. vannamei* juveniles was mainly excreted as ammonia, with only very small amounts of urea and uric acid that might be negligible. The excretory nitrogen is converted into energy using the equivalent of 24,830 J · g⁻¹ N for nitrogen excreted (Elliott 1976). Hence, the energy lost as U can be calculated using the equation:

$$U = (C_N - G_N - F_N - E_N) \times 24,830$$

where C_N , G_N , F_N , and E_N represent the nitrogen contents of diet, shrimp, feces, and molt, respectively. The nitrogen contents of diet, shrimp, feces, and molt were measured with a PE-240 element analyzer (Beijing NCS Analytical Instrument Company, Beijing, China). The initial nitrogen contents of the shrimp were assumed to be equal to the average nitrogen value of the shrimp sacrificed at the beginning of the experiment.

The energy lost as R can be calculated using the equation:

$$R = C - G - F - E - U$$

Statistical Analysis

Data from each treatment were subject to a two-way ANOVA. When overall differences were significant at less than the 5% level, Tukey's test was used to compare the mean values between individual treatments. The square-root transformation of the sine-arc before analyzing the values given in percentages was used. Statistical analysis was performed using SPSS (statistic package for social science) 10.0.

RESULTS

Growth

All shrimps survived at 4, 8, and 16‰, whereas no shrimps survived in tap water (about 0.2‰ salinity) at the end of the experiment, irrespective of dietary carbohydrate (CBH). Hence, analysis of the respective dependable variable of shrimps maintained in tap water was excluded from the experiment except for concerning the survival. Within the range of 1–2‰, the survival increased with increasing dietary CBH from 15.47% to 41.00%, and at each CBH level, the survival in shrimps maintained at 2‰ was slightly higher than those maintained at 1‰. At each dietary CBH level, the specific growth rate (SGR), food consumption (FC), and food efficiency (FE_f) generally increased with increasing salinity from 1‰ to 16‰, and the highest values were obtained in those maintained at 16‰ (Table 2 and Fig. 1).

A two-way ANOVA showed that salinity, dietary CBH, and the interaction between salinity and dietary CBH had significant effects on survival, SGR, FC, and FE_f, but none for absorption efficiency (AE). The relationship between SGR, FC or FE_f, salinity, and dietary CBH is shown in Table 3.

In the 16‰ groups, SGR and FC were similar in shrimps fed 29.15% and 15.47% dietary CBH, whereas those fed 41.00% dietary CBH exhibited a significantly lower value. In the 1–8‰ groups, SGR and FC in shrimps fed 29.15% dietary CBH were significantly higher as compared with other treatments (Fig. 1 and Table 2). The relationship between SGR and dietary CBH is shown in Table 4. Quadratic regression analysis indicated that the optimal CBH corresponding to maximum SGR decreased with increasing salinity from 1‰ to 16‰ and were 29.87%, 27.59%, 26.85%, 26.25%, and 22.29% at salinities of 1‰, 2‰, 4‰, 8‰, and 16‰, respectively.

TABLE 2.

The effects of salinity and dietary carbohydrate levels on growth, food consumption, food efficiency and absorption efficiency of *L. vannamei* juveniles.

Treatment		IW (g)	FW (g)	Survival (%)	FC (g)	FE _f (%)	AE (%)
Salinity (‰)	Carbohydrate (%)						
1	15.47	0.642 ± 0.002	1.779 ± 0.063 ^c	41.67 ± 14.43 ^b	2.613 ± 0.156 ^d	43.50 ± 0.45 ^h	84.92 ± 0.99
	29.15	0.643 ± 0.002	2.560 ± 0.052 ^c	50.00 ± 25.00 ^b	3.437 ± 0.070 ^b	55.77 ± 0.33 ⁱ	84.42 ± 0.90
	41.00	0.642 ± 0.004	2.058 ± 0.076 ^d	83.33 ± 14.43 ^a	2.700 ± 0.174 ^d	52.50 ± 0.61 ^g	85.21 ± 1.30
2	15.47	0.640 ± 0.004	2.240 ± 0.091 ^d	66.67 ± 14.43 ^{ab}	2.713 ± 0.163 ^d	58.98 ± 0.55 ^{cd}	85.41 ± 0.98
	29.15	0.636 ± 0.004	2.693 ± 0.017 ^{bc}	83.33 ± 14.43 ^a	3.523 ± 0.093 ^b	58.39 ± 0.94 ^{dc}	86.13 ± 0.90
	41.00	0.636 ± 0.007	2.130 ± 0.038 ^d	100.00 ± 0.00 ^a	2.713 ± 0.042 ^d	55.03 ± 0.35 ⁱ	84.43 ± 1.11
4	15.47	0.632 ± 0.003	2.598 ± 0.058 ^{bc}	100.00 ± 0.00 ^a	3.277 ± 0.090 ^{bc}	60.02 ± 0.87 ^{cd}	85.52 ± 1.01
	29.15	0.633 ± 0.003	3.386 ± 0.097 ^a	100.00 ± 0.00 ^a	4.403 ± 0.185 ^a	62.54 ± 0.43 ^b	86.29 ± 1.04
	41.00	0.631 ± 0.006	2.235 ± 0.059 ^d	100.00 ± 0.00 ^a	2.823 ± 0.105 ^{cd}	56.79 ± 0.35 ^{ef}	85.18 ± 1.22
8	15.47	0.636 ± 0.002	2.779 ± 0.121 ^{bc}	100.00 ± 0.00 ^a	3.543 ± 0.224 ^b	60.51 ± 0.54 ^c	86.13 ± 0.88
	29.15	0.637 ± 0.003	3.447 ± 0.097 ^a	100.00 ± 0.00 ^a	4.420 ± 0.200 ^a	63.60 ± 0.76 ^{ab}	86.12 ± 0.86
	41.00	0.634 ± 0.002	2.267 ± 0.074 ^d	100.00 ± 0.00 ^a	2.873 ± 0.161 ^{cd}	56.87 ± 0.72 ^{ef}	85.44 ± 1.14
16	15.47	0.637 ± 0.002	3.487 ± 0.106 ^a	100.00 ± 0.00 ^a	4.390 ± 0.217 ^a	64.95 ± 0.81 ^a	85.51 ± 1.01
	29.15	0.636 ± 0.004	3.483 ± 0.102 ^a	100.00 ± 0.00 ^a	4.450 ± 0.177 ^a	63.98 ± 0.35 ^{ab}	86.50 ± 1.01
	41.00	0.641 ± 0.005	2.811 ± 0.103 ^b	100.00 ± 0.00 ^a	3.653 ± 0.214 ^b	59.42 ± 0.72 ^{cd}	85.53 ± 0.94

Means in the same column sharing a common superscript letter were not significantly different ($P > 0.05$) as determined by Tukey's test.

IW, initial weight; FW, final weight; FC, food consumption; FE_f, food efficiency; AE, absorption efficiency; values are means of three groups of *L. vannamei* juveniles, with 4 shrimp per group ($n = 4$).

Energy Budget

The pattern of energy allocation varied with salinity and dietary CBH, in which 67.90–80.24% of consumed energy was lost through respiration, 7.25–18.14% invested in growth, 5.21–5.99% for feces, 3.87–8.73% for excretion, and only 0.69–0.73% for exuviae (Table 5). At each dietary CBH level with increases of salinity within the range of 1–16‰, the consumed energy and the percentage of consumed energy invested in growth (%C) exhibited increasing trends while that lost in respiration and excretion (%C) exhibited decreasing trends. At 16‰, the percentages of respiration and excretion (%C) were lowest of all while the consumed energy was highest of all; thus energy conversion efficiency was highest of all.

A two-way ANOVA showed that salinity, dietary CBH, and the interaction between salinity and dietary CBH had significant effects on growth, respiration, and excretion (%C), but none for feces and exuviae (%C). The relationship between growth, respiration or excretion (%C), salinity, and dietary CBH is shown in Table 3.

At 1–8‰, the percentage of consumed energy deposited in growth (%C) was significantly higher in shrimps fed 29.15% CBH as compared with other treatments, whereas at 16‰, the significantly higher value was in those fed 15.47% CBH (Table 5). The relationship between growth (%C) and CBH is shown in Table 4. Quadratic regression analysis indicated that the optimal CBH corresponding to maximum percentage in growth (%C) decreased with increasing salinity from 1‰ to 16‰ and was 29.36%, 26.75%, 26.50%, 26.40%, and 18.43% at salinities of 1‰, 2‰, 4‰, 8‰, and 16‰, respectively.

DISCUSSION

Salinity Effect on Growth of *L. vannamei* juveniles

A reduction in salinity can significantly affect the survival and growth of penaeid shrimps, although they possess the ability to tolerate low salinities (Huang 1983, Bray et al. 1994). Galli & Stern (1998) reported that the survival of *L. vannamei* was drastically reduced when salinity decreased below 0.18‰. Bray et al.

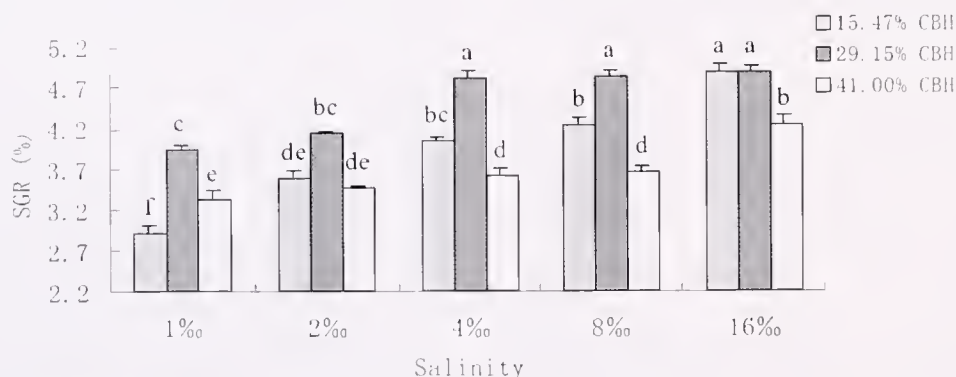


Figure 1. The effects of salinity and dietary carbohydrates (CBH) on specific growth rate (SGR) of *L. vannamei* juveniles; values are means of three groups of *L. vannamei* juveniles, with 4 shrimp per group ($n = 4$); means with different letters are significantly different ($P < 0.05$), and bars indicate standard errors of the means.

TABLE 3.

The relationship between specific growth rate, food consumption, food efficiency or the percentage of consumed energy allocated to growth, respiration or excretion (C%), salinity, and dietary carbohydrate.

	R ²
SGR = $0.391 + 0.133S + 0.258CBH - 0.004516CBH^2 - 0.002142S \cdot CBH$	0.836
FC = $0.986 + 0.141S + 0.323CBH - 0.005733CBH^2 - 0.002121S \cdot CBH$	0.867
FE _g = $35.017 + 1.229S + 1.492CBH - 0.02498CBH^2 - 0.02237S \cdot CBH$	0.583
G/C = $0.01177 + 0.634S + 0.921CBH - 0.01593CBH^2 - 0.009113S \cdot CBH$	0.745
R/C = $81.113 - 0.575S - 0.597CBH + 0.01285CBH^2 + 0.008489S \cdot CBH$	0.816
U/C = $10.831 - 0.06321S - 0.162CBH + 0.0009724S \cdot CBH$	0.986

S, salinity; CBH, carbohydrate; SGR, specific growth rate; FC, food consumption; FE_g, food efficiency; G/C, the percentages of consumed energy allocated to growth; R/C, the percentages of consumed energy allocated to respiration; U/C, the percentages of consumed energy allocated to excretion (%).

(1994) reported that *L. vannamei* juveniles had growth optima between 5‰ and 15‰. Currently, no shrimps survived in tap water (about 0.2‰ salinity), the survival increased with increasing salinity from 1‰ to 2‰, and they grew well in salinities ≥4‰, indicating the limited capability for *L. vannamei* juveniles to tol-

TABLE 4.

The relationship between specific growth rate or the percentage of consumed energy invested in growth (C%) and dietary carbohydrate.

Salinity (‰)		R ²	Max _{CBH} (%)
1	SGR = $-50.039CBH^2 + 29.896CBH - 0.517$	0.969	29.87
2	SGR = $-37.783CBH^2 + 20.845CBH + 1.2562$	0.971	27.59
4	SGR = $-60.59CBH^2 + 32.535CBH + 0.4569$	0.984	26.85
8	SGR = $-56.578CBH^2 + 29.709CBH + 0.9711$	0.975	26.25
16	SGR = $-20.895CBH^2 + 9.316CBH + 3.9162$	0.932	22.29
1	G/C = $-328.32CBH^2 + 192.76CBH - 14.716$	0.999	29.36
2	G/C = $-159.91CBH^2 + 85.539CBH + 3.1997$	0.988	26.75
4	G/C = $-116.72CBH^2 + 61.854CBH - 7.6482$	0.985	26.50
8	G/C = $-133.54CBH^2 + 70.511CBH - 7.374$	0.985	26.40
16	G/C = $-58.178CBH^2 + 21.439CBH + 16.214$	0.984	18.43

CBH, carbohydrate; SGR, specific growth rate; G/C, the percentage of consumed energy invested in growth (C%); Max_{CBH}, the optimal carbohydrate corresponding to maximum specific growth rate or the percentage of growth (C%).

erate very low salinities. It was apparent from the experiment, however, that salinity had significant effects on both growth and food efficiency. At each dietary carbohydrate level, the maximum specific growth rate and food efficiency were obtained in shrimp maintained at 16‰ and diminished with a reduction in salinity; the shrimps maintained at 1–2‰ exhibited significantly lower values. Hence, at salinities ≤16‰, decreasing salinity significantly affected the growth rate of *L. vannamei* juveniles and even endangered their survival.

The growth rate of the shrimp seems to have a direct relation with food consumption, changes of which may reflect the adaptation to various environmental conditions. Previous studies had shown that holding penaeid shrimps at salinities near isosmotic points could result in an increase of food consumption (Staples & Heales 1991). Rosas et al. (2001a) reported that *L. vannamei* juveniles maintained at 15‰ salinities consumed 1.69 times more food than those at 5‰. Similarly, in the present study, food consumption generally increased with increasing salinity within the range of 1–16‰ with the maximum value at 16‰, which coincided with the salinity at which the highest specific growth rate was observed, indicating that at salinities ≤16‰, holding *L. vannamei* juveniles at salinities closer to isosmotic points could result in increased appetite.

The isosmotic point for penaeids ranged from 23 to 30 ppt (Dall 1981; Castille & Lawrence 1981). It is generally believed that shrimps near the isosmotic point expend the least energy on respiration and excretion, and so they exhibit the best growth rate and the most efficient energy conversion (Panikkar 1968). By contrast, when shrimps are under anisomotic conditions, additional energy is required to meet the osmotic adjustments, which generally increase as a function of deviations in salinity from the isosmotic zone; that is, when the concentration gradient between ambient salinity and body fluids increases, more energy is expended for recovery of homeostasis and, as a result, less energy is available for growth (Zhang & Dong 2002; Bindu & Diwan 2002). In the present study, as the range of salinity tested deviated from the isosmotic point, the energetic demand on maintaining homeostasis, i.e., the percentages of energy lost to respiration and excretion (C%), increased with decreasing salinity from 16‰ to 1‰.

The results obtained in our work provide evidence that the growth differences between salinities are primarily the consequence of variations in both food consumption and energy allocation. Food consumption and energy gain (the percentage of energy invested in growth, C%) of *L. vannamei* juveniles appeared to increase with increased salinity within the range of 1–16‰, whereas total metabolic expenditure (the percentages of energy lost to respiration and excretion, C%) displayed inverse trends with salinity. Thus, the enhanced growth of *L. vannamei* juveniles with increasing salinity from 1‰ to 16‰ was mainly due to the significantly increased food consumption associated with the significantly decreased proportion of energetic cost for osmotic regulation. At 16‰, the food consumption was highest of all while the proportion of energy losses was least of all, and consequently, the growth rate was highest of all. By contrast, previous investigations suggested that the depression of growth for the shore crab *Carcinus maenas*, the common carp *Cyprinus carpio*, and the blue crab *Callinectes sapidus* under hypo-osmotic stresses could not be explained with enhanced metabolic demands; rather, this result was a consequence of a significantly reduced assimilation capability (Guerin & Stickle 1992, 1995; Wang et al. 1997; Anger et al. 1998). In the present study, salinity had no significant effects on

TABLE 5.

The effects of salinity and dietary carbohydrate levels on energy budget of *L. vannamei* juveniles.

Treatment		C allocation ² (%)					
Salinity (‰)	Carbohydrate (%)	C ¹ (kJxg ⁻¹ × d ⁻¹)	G ³	R ³	U ³	F ³	E ³
1	15.47	1.33 ± 0.08 ^d	7.25 ± 0.08 ^k	77.79 ± 0.27 ^b	8.73 ± 0.01 ^a	5.53 ± 0.36	0.70 ± 0.02
	29.15	1.72 ± 0.04 ^b	13.58 ± 0.08 ^a	74.05 ± 0.33 ^{de}	5.87 ± 0.02 ^c	5.79 ± 0.38	0.71 ± 0.03
	41.00	1.34 ± 0.09 ^d	9.13 ± 0.06 ⁱ	80.24 ± 0.29 ^a	4.40 ± 0.03 ^b	5.52 ± 0.31	0.71 ± 0.01
2	15.47	1.38 ± 0.08 ^d	12.61 ± 0.09 ^b	73.01 ± 0.36 ^{cd}	8.22 ± 0.02 ^b	5.45 ± 0.37	0.71 ± 0.02
	29.15	1.77 ± 0.05 ^b	14.55 ± 0.26 ^f	73.62 ± 0.35 ^{cd}	5.84 ± 0.03 ^c	5.28 ± 0.34	0.70 ± 0.02
	41.00	1.35 ± 0.02 ^d	11.39 ± 0.13 ⁱ	77.62 ± 0.51 ^b	4.30 ± 0.04 ⁱ	5.99 ± 0.43	0.70 ± 0.01
4	15.47	1.66 ± 0.05 ^{bc}	14.42 ± 0.23 ^f	71.40 ± 0.41 ^b	7.98 ± 0.03 ^c	5.48 ± 0.38	0.71 ± 0.02
	29.15	2.21 ± 0.09 ^a	15.76 ± 0.12 ^d	72.62 ± 0.41 ^{fg}	5.65 ± 0.04 ^d	5.26 ± 0.40	0.72 ± 0.02
	41.00	1.40 ± 0.05 ^d	13.39 ± 0.04 ^g	76.10 ± 0.39 ^c	4.07 ± 0.03 ^j	5.74 ± 0.44	0.69 ± 0.00
8	15.47	1.80 ± 0.11 ^b	15.09 ± 0.13 ^c	70.94 ± 0.41 ^b	7.96 ± 0.04 ^c	5.31 ± 0.33	0.73 ± 0.01
	29.15	2.22 ± 0.10 ^a	16.58 ± 0.20 ^e	71.76 ± 0.33 ^{gh}	5.61 ± 0.02 ^g	5.34 ± 0.43	0.71 ± 0.02
	41.00	1.43 ± 0.08 ^d	13.83 ± 0.17 ^g	75.79 ± 0.20 ^c	4.04 ± 0.02 ^j	5.63 ± 0.27	0.70 ± 0.01
16	15.47	2.23 ± 0.11 ^a	18.14 ± 0.26 ^a	67.90 ± 0.20 ⁱ	7.65 ± 0.02 ^d	5.60 ± 0.39	0.69 ± 0.01
	29.15	2.23 ± 0.09 ^a	17.52 ± 0.09 ^b	71.01 ± 0.32 ^h	5.55 ± 0.01 ^g	5.21 ± 0.40	0.72 ± 0.01
	41.00	1.81 ± 0.11 ^b	15.22 ± 0.21 ^c	74.48 ± 0.40 ^d	3.88 ± 0.03 ^k	5.70 ± 0.44	0.70 ± 0.02

Means in the same column sharing a common superscript letter were not significantly different ($P > 0.05$) as determined by Tukey's test.¹ C, the consumed energy; values are means of three groups of *L. vannamei* juveniles, with 4 shrimp per group ($n = 4$).² The energy values expressed as the percentages of consumed energy.³ G, growth; R, respiration; U, excretion; F, feces; E, exuviae; values are means of three groups of *L. vannamei* juveniles, with 4 shrimp per group ($n = 4$).

absorption efficiency and the percentage of fecal production (%C), indicating the different bioenergetic mechanisms by interspecies.

Carbohydrate Effect on Growth of *L. vannamei* Juveniles at Low Salinities

Carbohydrate is considered the most economical source of dietary energy in penaeid shrimp. Previous investigations on a variety of penaeid species indicated an improved growth on diets with an adequate dietary carbohydrate supply. Ali (1982) reported that the survival, growth, and food conversion efficiency of juvenile *P. indicus* increased with an increase in the dietary carbohydrate level from 10–40%. Rosas et al. (2001b) reported that the maximum growth rate for *L. vannamei* juveniles occurred between 6% and 23% of dietary carbohydrate.

It is generally believed that more energy is expended for osmoregulation by aquatic animals subjected to hypo-osmotic environments as compared with those maintained under isosmotic conditions (Panikkar 1968). Hence, we speculated that maximizing the carbohydrate content relative to protein in diets might promote the growth of penaeid shrimp maintained at low salinities, based on

the assumption that diets were formulated to be isoenergetic. In the present study, dietary carbohydrate had significant effects on growth, food consumption, and energy gain (the percentage of energy invested in growth, C%). At 1–8‰, the specific growth rate in shrimps fed 15.47% dietary carbohydrate was significantly lower than those fed 29.15% dietary carbohydrate; by contrast, at 16‰, the specific growth rate in shrimps fed 15.47% dietary carbohydrate was not significantly different from those fed 29.15% dietary carbohydrate. The responses of food consumption and energy gain (C%) to dietary carbohydrate were similar to that of the specific growth rate. These results confirmed that carbohydrate was an important source of energy for *L. vannamei* juveniles maintained at low salinities, and an adequate level of carbohydrate provided in diets could not only significantly improve growth performance of *L. vannamei* juveniles, but also have a protein sparing action.

ACKNOWLEDGMENTS

This study was funded by the Chinese National Agricultural Development Project (Grant no. K2002-15) and the Science Research Council of Shandong Province, China (Grant no. 041656).

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PARAPENAEON CONSOLIDATUM (ISOPODA: BOPYRIDAE) AND THE RELATIVE GROWTH AND REPRODUCTION OF METAPENAEOPSIS DALEI (DECAPODA: PENAEIDAE) IN SOUTH KOREA

JUNG HWA CHOI,¹* GLEN JAMIESON,¹ KYEONG HO HAN² AND SUNG YUN HONG³

¹Fisheries and Oceans Canada Pacific Biological Station, Nanaimo, BC V9T 6N7, Canada;

²Department of Aquaculture, Yosu National University, Yosu-shi 550-749, Korea; and ³Department of Marine Biology, Pukyong National University, Busan 608-737, Korea

ABSTRACT This study describes the prevalence of a parasitic bopyrid isopod, *Parapenaeon consolidatum*, on *Metapenaeopsis dalei* and its effect on host growth and reproduction. The prevalence of *P. consolidatum* increased with shrimp size, from 27.3% at 5 mm CL to 68.4% at 16 mm CL. Lengths of female bopyrids and that of their hosts were positively correlated. This indicated that the bopyrid stays continuously with its host and is not detached when the host molts. Morphometric analyses indicated that parasitized male and female shrimp have slightly lower body weight and length than nonparasitized shrimp of the same carapace length. The gonadosomatic index was higher for nonparasitized than for parasitized female shrimp. Growth of the petasma, the copulatory organ of male shrimp, was significantly affected by the parasite.

KEY WORDS: *Metapenaeopsis dalei*, *Parapenaeon consolidatum*, growth, reproduction

INTRODUCTION

The kishi velvet shrimp, *Metapenaeopsis dalei* (Rathbun), is distributed throughout the coastal waters of Korea, Japan, and China in sandy muddy substrates at 30–130 m depth (Kim 1977, Hayashi 1992). With seasonal warming of the waters starting in April, the shrimp begin to migrate from deeper water to the coastal area. They are exploited commercially in Korea from April to October, using shrimp trawl and stow nets (Cha 1997). *Metapenaeopsis dalei* exhibit higher burrowing rates than other penaeid shrimp (Sakaji 1995). Owens (1987) reported that the bopyrid isopod *Parapenaeon consolidatum* Richardson is a parasite on *M. dalei* from Japanese waters. However, little is known about the effects of this parasite on growth and reproduction of its host.

Bopyrids live on shrimp, brachyurans, and anomurans and cause a conspicuous bulge of the branchiostegite (Dall et al. 1990). Owens and Glazebrook (1985) showed that bopyrids on penaeids grow with their hosts and have a similar longevity, although there is some loss of parasites as the prawns approach asymptotic length. Some studies hypothesized a relationship between a bopyrid and its host because of a highly positive correlation between the sizes of female bopyrids and their shrimp hosts (see Beck 1980; Schuldt & Rodrigues Capitulo 1985). Cash and Bauer (1993) reported that *Probopyrus pandalicola* (Packard) remain on and grow synchronously with *Palaemonetes pugio* Holthuis; video recording has shown that reproductive activities of the parasites are synchronized with host molting.

Both growth and reproduction of shrimps can be affected by bopyrids. Abu-Hakima (1984) showed that parasitized male *Penaeus semisulcatus* De Haan grew to the same size as nonparasitized females but that host reproduction was totally inhibited. She also showed that a petasma, the male copulatory organ, was not formed in parasitized male shrimp. Chu & Leong (1996) indicated that parasitized male and female shrimp have slightly lower average body weights than nonparasitized shrimps of the same carapace length. Dawson (1958) reported that in the Arabian Gulf, only

P. semisulcatus larger than 100 mm TL (total length) were parasitized with bopyrids. Mathews et al. (1988) found low parasite prevalence among *P. semisulcatus* of 14–18 mm CL (carapace length) by *Epipenaeon elegans* Chopra; prevalence increased up to a prawn size of around 28 mm CL, after which the rate fell, apparently because of loss of the parasite. Some bopyrid parasites alter the appearance or behavior of the host whereas others are asymptomatic.

This study provides additional information on the prevalence of *P. consolidatum* in relation to host size, the effects of bopyrid parasitism on the growth and reproduction of parasitized shrimp, and the relationship between the host shrimp and bopyrid breeding rate.

MATERIALS AND METHODS

Shrimps were collected monthly from September 1998 to June 2000 with a research shrimp beam trawl (mesh size: 9.3 × 9.3 mm) in waters around Yongchodo, South Korea (128°40'E, 34°30'N). The study area was sandy mud in the channel between Hansando and Yongchodo at 20–30 m depth. During this study, water temperature varied from 6.7 to 25.8°C (Fig. 1). Samples were fixed immediately in neutralized formaldehyde for 1–2 days and were then preserved in 70% alcohol.

Specimens were sorted and sexed. Parasitized shrimps were detected by an obvious deformation of their carapace. Bopyrids were removed, and the females were classified as ovigerous or nonovigerous. In the shrimp, carapace length (CL, from posterior orbital margin to the posterior edge of carapace in the mid-dorsal line) and body length (BL, from the tip of the rostrum to the end of the telson) of shrimp were measured with a caliper to the nearest 0.1 mm. Shrimp body weight (BW) was determined to the nearest 0.01 g after the shrimp was blotted dry. In males, the petasma length (PL, from the tip of the lobe to the end of the petasma) was measured to the nearest 0.01 mm under a dissection microscope. In parasites, body lengths (from the anterior margin of the head to the posterior margin of the pleotelson) of female (FPL) and male (MPL) bopyrids were measured under a dissection microscope to the nearest 0.01 mm.

The effects of the parasites on host log transformed BW, BL, and PL (reproduction) were determined statistically by analysis

*Correspondence present address and E-mail: Korea Inter-University Institute Ocean Sciences, Pukyong National University, Busan 608-737, Korea; shrimpchoi@hanmail.net

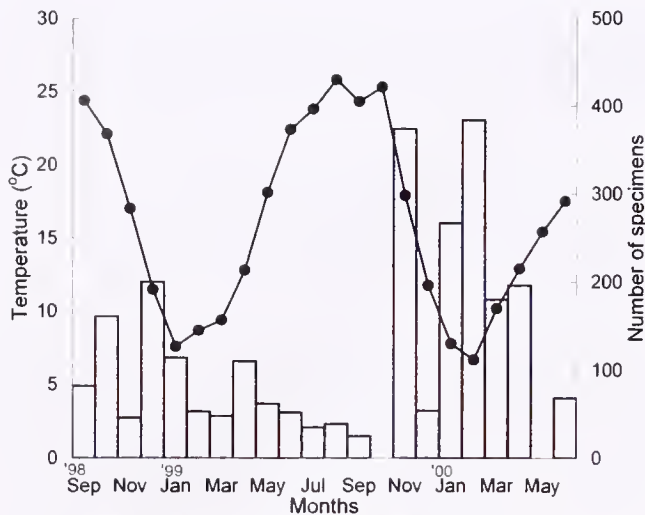


Figure 1. Monthly variation of seawater surface temperature (line) and number of specimens (bars) at the sampling sites.

with CL as covariance (ANCOVA) using Minitab version 2.0 (Sokal & Rohlf 1995). Analyses were based on individuals with no visibly developed gonads (CL < 12.09 mm; Choi 2001), as gonad development alters the length-weight relationship of both female and male shrimp (Chu & Leong 1996).

RESULTS

Prevalence

A total of 2550 specimens of *M. dalei* were caught (Fig. 1). Females were consistently more abundant in all size classes; the overall proportion of females was 53.1% but varied with shrimp size. The proportion of females sharply increased from 50–100% for size classes >16 mm CL (Fig. 2). Forty-eight percent of both the 1360 female shrimp and the 1190 male shrimp were parasitized

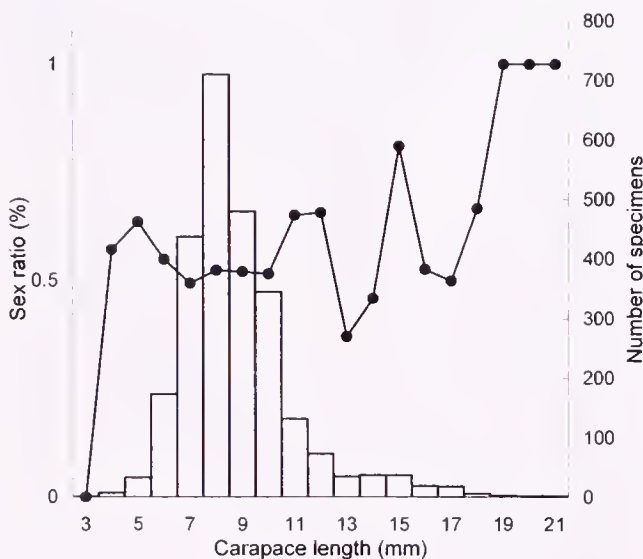


Figure 2. Size-specific female sex ratio (line) and number of *Metapenaeopsis dalei* (bars) caught at Yongchodo from September 1998 to June 2000. Sex ratio = (number of female specimens/total number of specimens) \times 100.

by *P. consolidatum*. The smallest parasitized female and male shrimp were 4.21 mm and 4.72 mm CL, respectively. The largest parasitized female and male shrimps were 21.25 mm and 18.44 mm CL, respectively. Female prevalence increased from 28.6% at 5 mm CL to 62.3% at 10 mm CL, leveled off between 11 and 12 mm CL, and sharply increased from 37.5% at 12 mm CL to 90% at 16 mm CL. Male prevalence increased from 25% at 5 mm CL to 59.5% at 9 mm CL, decreased between 10 and 11 mm CL, and increased from 43.4% at 11 mm CL to 100% at 15 mm CL. Parasitism incidence for females and males >16 mm CL and >15 mm CL, respectively, dropped sharply (Fig. 3). Overall, parasitism occurred early in the life of the host and until shrimp reached 11 mm CL, prevalence was the same in both sexes. However, between 12 mm and 15 mm CL, prevalence among males was higher than among females.

Host-parasite Relationship

Female *P. consolidatum* filled most of and were tightly lodged within the host's branchial chamber, producing a characteristic bulge in the host's branchiostegite. Male parasites oriented perpendicularly to the female's body axis on the ventral side of the female's abdomen. Regression analysis showed a significant relationship between FPL and CL (Fig. 4). The relationship was similar for female and male hosts (ANCOVA intercept: $F = 3.08$, $df = 1,254$, $P = 0.081$). The relationship between FPL and MPL was also significant (Fig. 5).

Host Growth Rate Effects

CL-BL regression slopes for parasitized and nonparasitized shrimp were significantly different for female shrimp (ANCOVA: male, $F = 3.72$, $df = 1,39$, $P = 0.06$; female, $F = 11.31$, $df = 1,71$, $P < 0.05$) (Fig. 6). Also, parasitized and nonparasitized shrimp had significantly different CL-BW regression slopes (ANCOVA: male, $F = 5.13$, $df = 1,114$, $P < 0.05$; female, $F = 10.24$, $df = 1,153$, $P < 0.05$) (Fig. 7).

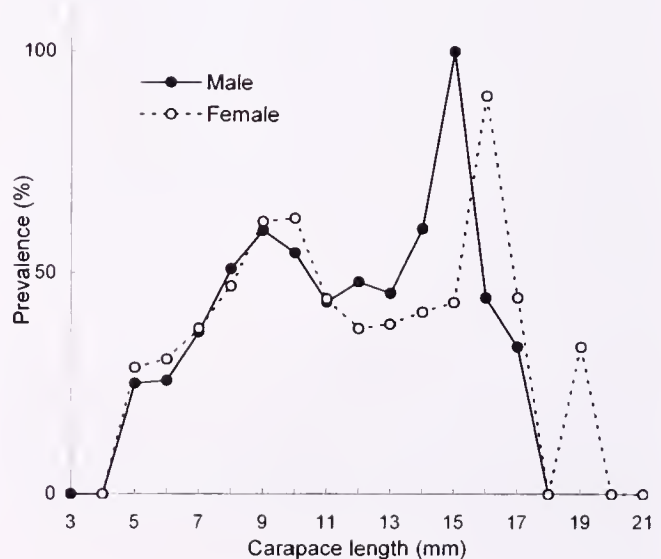


Figure 3. Size-specific prevalence (%) of the endoparasite *Parapenaeon consolidatum* on *Metapenaeopsis dalei* caught from Yongchodo from September 1998 to June 2000. Prevalence = number of parasitized specimens/total number of specimens \times 100.

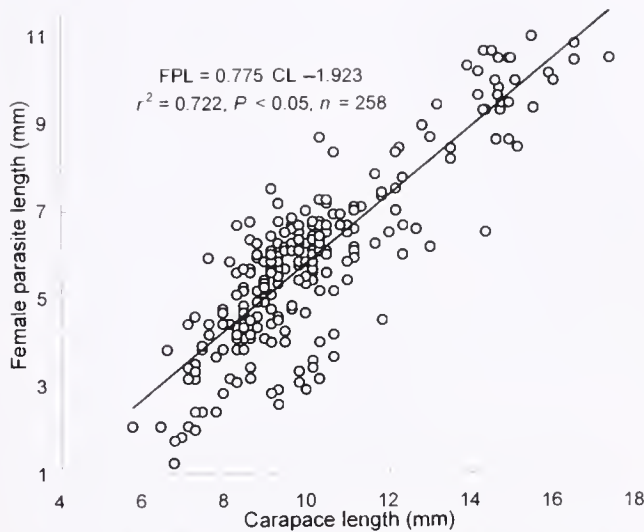


Figure 4. Relationship between carapace length of *Metapenaeopsis dalei* and total length of its female parasite (*Parapenaeon consolidatum*) from Yongchodo between January and December 1999.

Host Reproduction Effects

There were no changes in the structure of the thelycum in parasitized female shrimps. In nonparasitized males, the petasma consisted of the typical complex of membranous folds and ridges in the endopodites of the first pairs of pleopods. In parasitized males, endopodites were only partially fused or more frequently were separate structures. The PL-CL regressions for nonparasitized and parasitized male shrimp had similar slopes (ANCOVA: $F = 2.56$, $df = 1,204$, $P > 0.05$) but different intercepts (ANCOVA: $F = 125.31$, $df = 1,205$, $P < 0.05$) (Fig. 8). Thus, the PL of parasitized male shrimp was smaller than that of nonparasitized male shrimp.

To investigate the influence of the bopyrid parasite on gonad development, the gonadosomatic index (GSI) of female shrimp

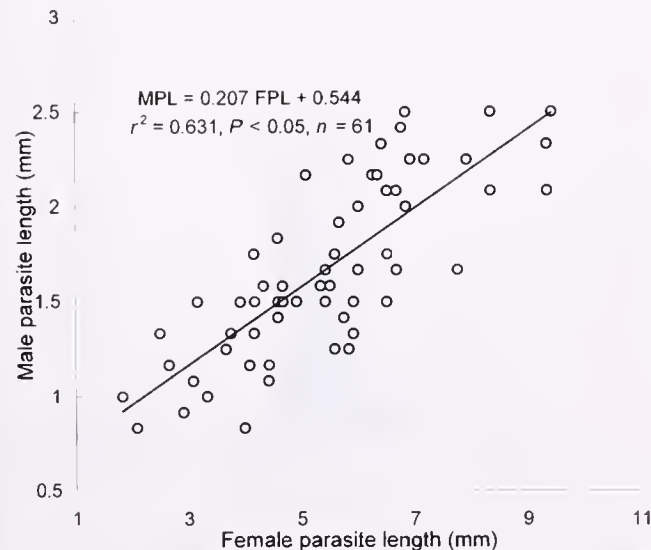


Figure 5. Relationship between female parasite and male parasite (*Parapenaeon consolidatum*) total lengths on *Metapenaeopsis dalei* from Yongchodo between January to December 1999.

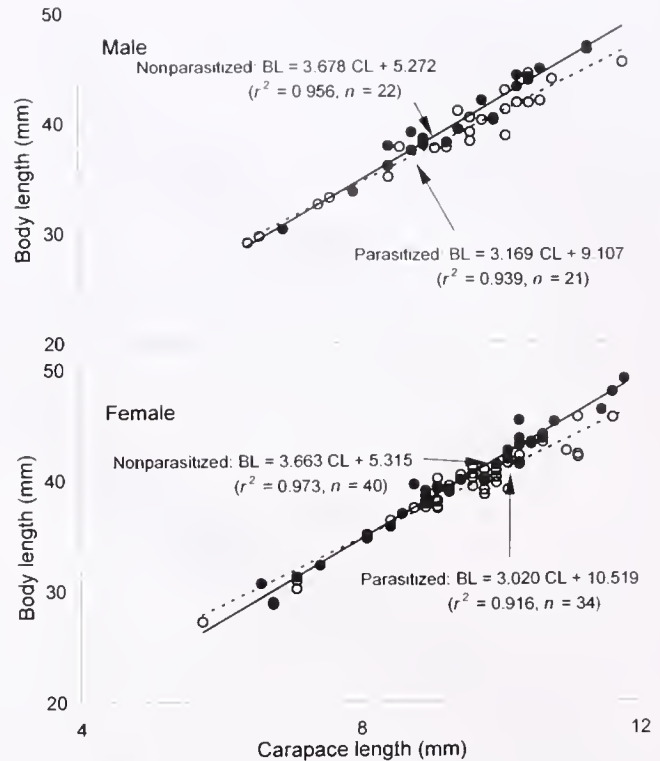


Figure 6. *Metapenaeopsis dalei* body length (BL) to carapace length (CL) relationships for parasitized and nonparasitized shrimp. Regression lines were calculated from the specimens caught from Yongchodo on November 1999. Open and solid circles represent parasitized and nonparasitized shrimp, respectively.

caught during the breeding season (July–August, Choi 2001) were compared with CL. GSI values of nonparasitized female shrimps were significantly larger than those of parasitized shrimps (Kruskal-Wallis Test: $H = 8.10$, $P < 0.01$) (Fig. 9).

Parasite Maturity

Size frequency distribution of female parasites is shown in Figure 10.

DISCUSSION

In this study, the 48% parasitism rate of *M. dalei* by the bopyrid *P. consolidatum* was much higher than the 0.24% reported for *Penaeus longistylus* Kubo parasitized by *Parapenaeon japonicum* (Thielemann) and *Parapenaeon prox. expansus* on Queensland's east coast (Courtney 1991). Prevalence fluctuated during the sampling year, as observed for other bopyrid-host pairs (Beck 1980, Somers & Kirkwood 1991). Seasonal fluctuations have been ascribed to differential migration patterns among parasitized and nonparasitized shrimp or to seasonal variations in the reproduction and mortality rates of hosts and parasites (Mathews et al. 1988; Torres Jordá & Roccatagliata 2002).

Roccatagliata & Lovrich (1999) reported that the prevalence of the parasite *Pseudione tuberculata* Richardson decreased with *Paralomis granulosa* (Jacquinot) size and suggested a high mortality rate for small parasitized *P. granulosa*. In contrast, we observed increasing parasitism in proportion with size for the smaller host size classes (3–9 mm CL), suggesting that *M. dalei* did not cause increasing mortality in the small size classes. We suggest the

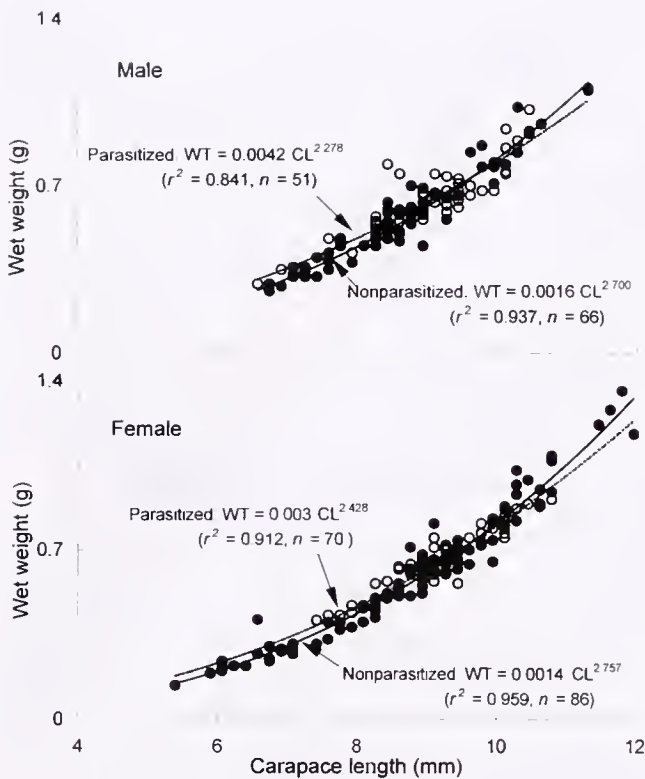


Figure 7. *Metapenaeopsis dalei* wet weight (WT) to carapace length (CL) relationships for parasitized and nonparasitized shrimp. Exponential growth curves were calculated from the specimens caught from Yongchodo on November 1999. Open and solid circles represent parasitized and nonparasitized shrimp, respectively.

decrease in prevalence between size classes 9 mm and 12 mm CL was due to a relatively higher host growth rate of these individuals during the study period. Choi (2001) reported that at these size classes, shrimp frequently molted during the summer. Somers &

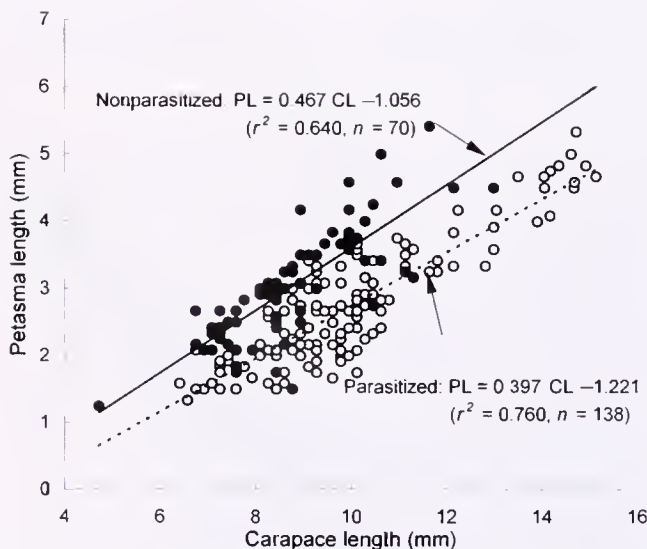


Figure 8. Regressions of petasma length (PL) to carapace length (CL) in male *Metapenaeopsis dalei*. Regression lines were calculated from the specimens caught from Yongchodo between January and December 1999.

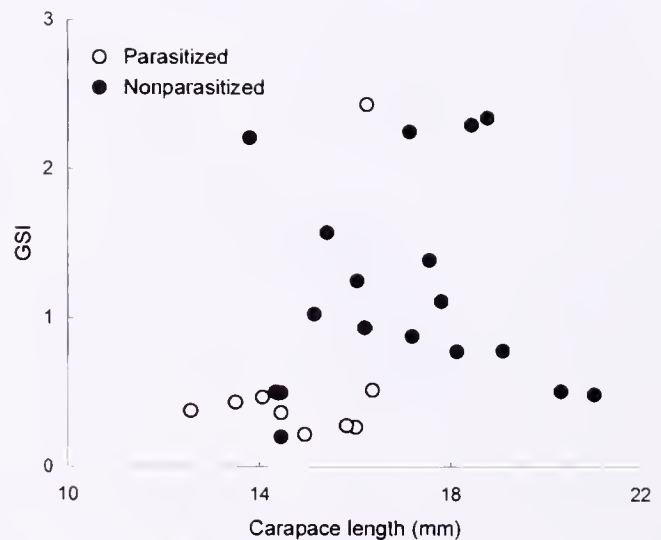


Figure 9. Gonadosomatic indices (GSI) relative to carapace length for parasitized and nonparasitized female of *Metapenaeopsis dalei* caught from Yongchodo between June and September 1999. GSI = (gonad wet weight/total body wet weight) \times 100.

Kirkwood (1991) showed that *P. semisulcatus* parasitized by *Epipenaeon ingens* Nobili may lose their bopyrids: of 22 parasitized prawns that were released, six had lost their bopyrids upon recapture two weeks later. Cash & Bauer (1993) gave further direct evidence of parasite loss in *P. pugio* parasitized by *P. pandalicola*. They noted that bopyrids were shed with the shrimp exuviae in three of the 112 moltings examined. Additional indirect evidence of parasite loss is from the size of maturity of female parasites, which begin spawning at 4–5 mm FPL (host size of ca. 9 mm CL). Cash & Bauer (1993) reported that *P. pandalicola* larvae escaped from the host, *P. pugio*, during the latter's molting period. They showed that epicaridium larvae were released from the host's branchial chamber several hours before the host molted. A de-

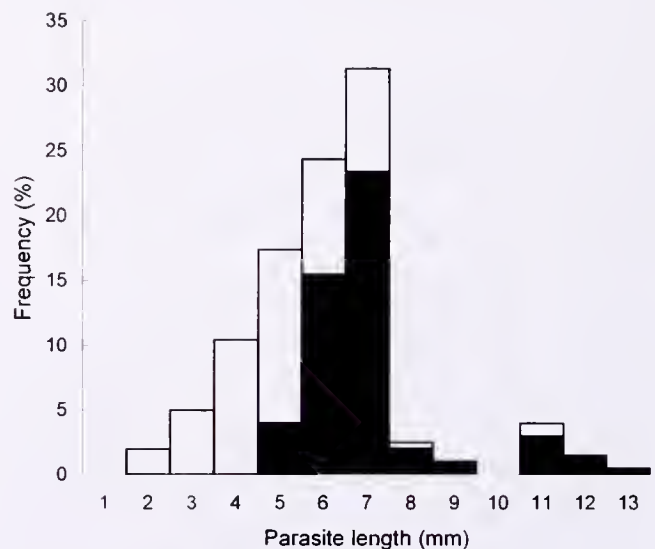


Figure 10. Size-frequency distribution of female parasite *Parapenaeon consolidatum* on *Metapenaeopsis dalei* caught from Yongchodo November 1999. Solid and open bars represent ovigerous and nonovigerous parasite females, respectively.

creasing parasitism with larger size classes of shrimp can also perhaps be explained by a shorter life span of parasitized shrimp versus that of nonparasitized shrimp.

The positive correlation of FPL of *P. consolidatum* with the CL of *M. dalei* (Fig. 4) suggests that the parasite attaches to small hosts and grows along with them. This should negatively affect host growth (Chu & Leong 1996). However, we found that with <8 mm CL size classes of parasitized shrimp, BL and WT averaged relatively larger on nonparasitized shrimp of the same CL. However, by the time they reached 8 mm CL, the ratios of BL and WT to CL were relatively similar for both groups of shrimp. This finding may be explained by two hypotheses. First, parasitized shrimp may initially consume more than nonparasitized shrimp and hence grow faster; the parasite may thus be influencing the behavior of the shrimp. Anderson (1977) reported that *P. pugio* parasitized by *P. pandalicola* ate more *Artemia salina* Linnaeus nauplii than nonparasitized shrimp. Second, *P. consolidatum* may preferentially select larger CL hosts at the time of infection, because a larger host might provide more space for bopyrid development and reproduction. The strategy of the parasite may thus be to select a host with the optimal growth and size. Either approach might be adaptive, because parasite size and fecundity are related to host size (see Baudoin 1975; Shearer 1977).

BL and WT of parasitized shrimp are smaller than with nonparasitized shrimps for >8 mm CL size classes. The increased breeding rate of female parasites on shrimp >8 mm CL may be indicative of a negative effect on shrimp by the parasite. Bopyrid isopods have evolved as branchial parasites on decapod crustacean hosts (cf., Markham 1975) and affect their host in a variety of ways through their high rate of ingestion of the host's hemolymph (Walker 1977). Nelson et al. (1986) showed that the energy expenditure of breeding bopyrid *Argeia pauperata* Stimpson females is more than that of its normal host, *Crangon franciscorum* Stimpson.

Parapenaeon consolidatum affects the genital growth of *M.*

dalei; the length of a parasitized shrimp's petasma is less than that on a nonparasitized shrimp. The length of the petasma normally increases directly with increasing CL (Burkenroad 1934). Some studies suggested that the petasma assists in the transfer of spermatophores to the female (Hudinaga 1942; King 1948). We suggest that in parasitized males, a smaller petasma length negatively affects sperm transfer and hence copulation success. In addition, our observation indicated that a few parasitized larger males had unfused or only a partly joined petasma. Dall (1958) defined male maturity of *Metapenaeus mastersii* (Haswell) (= *Metapenaeus bennettiae* Racek & Dall) by the presence of a fully developed petasma. Tuma (1967) reported that small *Penaeus merguensis* De Man males had a longitudinally constricted petasma on their pleonic endopodites; when mature, the end of endopodites had joined to form the petasma. Thus, *P. consolidatum* affects the growth of the petasma in *M. dalei*, which likely negatively affects male shrimp reproductive ability.

CONCLUSION

Our main conclusions are that *M. dalei* has an exceptionally high rate of parasitism by *P. consolidatum*. Parasitism proportion increased with host shrimp size. Selection of a larger host may provide more branchial chamber space for the parasite, or *M. dalei* may grow faster because of parasite induced hunger, because other studies have shown that parasitized shrimp eat more food than do nonparasitized shrimp. Because the parasite negatively effects growth of the host's genital organ and development of gonads, it may be reducing population fecundity because a large portion of the male population consequently cannot effectively reproduce.

ACKNOWLEDGMENTS

The authors thank Dr. Matrn Torres Jordá (Universidad de Buenos Aires, Argentina) and Dr. Daniel Roccatagliata (Universidad de Buenos Aires, Argentina) for critical reading and comments on the manuscript.

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THE EFFECT OF STARVATION ON REFEEDING, DIGESTIVE ENZYME ACTIVITY, OXYGEN CONSUMPTION, AND AMMONIA EXCRETION IN JUVENILE WHITE SHRIMP *LITOPENAEUS VANNAMEI*

LAURA I. COMOGLIO,^{1,*} GABRIELA GAXIOLA,² ANA ROQUE,^{3,4} GERARD CUZON⁵ AND OSCAR AMIN¹

¹Centro Austral de Investigaciones Científicas (CADIC–CONICET), Ushuaia, Tierra del Fuego, Argentina; ²Facultad de Ciencias, UNAM, México; ³Centro de Investigación en Alimentación y Desarrollo, Unidad en Acuicultura y Manejo Ambiental, Mazatlán, Sinaloa, México; ⁴IRTA-Centre d'Aquicultura Sant Carles de la Rapita, Spain; ⁵Centre Océanologique du Pacifique, Tahiti, IFREMER, Francia

ABSTRACT Juveniles of the white shrimp *Litopenaeus vannamei* were kept without food for between 0 to 15 days to evaluate the impact of starvation on physiologic state (oxygen consumption, poststarvation refeeding index, nitrogen excretion, and O:N ratio) and digestive enzymes activity. Physiologic changes were found after 6 days of fasting, and refeeding ability declined as a result. Nevertheless, the shrimp were able to survive 16 days without food. Starvation caused metabolism to drop progressively toward a basal level ($21 \text{ J} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$) and a decrease in the rate of ammonia excretion, because of the catabolism of amino acids from soluble protein in the hepatopancreas. This decrease led to an increase in digestive enzymes specific activity (U/mg protein). But, expressed as total U, all digestive enzyme activities decreased in the absence of substrate from 0.016 to 0.007 U/hepatopancreas (HP) for α -amylase and 2.58 to 0.63 U/HP for total trypsin. *L. vannamei* juveniles showed a true physiologic adaptation mechanism to food deprivation: no changes in body weight but loss in hepatosomatic index, no exuviations, including the utilization of HP soluble proteins (a drop from 269 to 53 mg/mL). After 10 days, a neoglycogenic pathway and the corresponding tissue enzymes activities seemed enhanced, and the animals derived all energetic substrates mainly from protein (O:N ratio of 17) to cover their metabolic costs. Estimates of basal metabolism (Hem) from the routine respiration rate per day (from 361 to 725 $\text{J} \cdot \text{g} \text{ ww}^{-1} \cdot \text{day}^{-1}$ through the 15-day starvation period), and loss of nonfecal energy (HxE) from the nitrogen excretion rate (varying from 39 to 57 $\text{J} \cdot \text{g} \text{ ww}^{-1} \cdot \text{day}^{-1}$ during the same period) were used in a bioenergetic partition model of a fasting juvenile, which indicated that the energetic requirement to survive without feeding was in the range of 418 and 771 $\text{J} \cdot \text{g} \text{ ww}^{-1} \cdot \text{day}^{-1}$ during the 15-day period of starvation.

KEY WORDS: digestive enzymes, *Litopenaeus vannamei*, metabolic rate, shrimp, starvation

INTRODUCTION

The Pacific white shrimp *Litopenaeus vannamei* is cultured in extensive, intensive, and semi-intensive systems and is, with *Litopenaeus stylirostris*, the most popular shrimp for aquaculture in Mexico and Central and South America. The state of Sinaloa on the northwest coast of Mexico, considered the most important agricultural producer in the country, has observed in recent years an important increase of aquaculture farms, from approximately 100 hectares of ponds in 1984 to 1850 hectares in 1998, with a growth rate of about 150 hectare/year. Nowadays, Sinaloa has more than 200 shrimp farms (about 75% of the national total) and produces around 10,000 tons yearly (65% of the national total) (Hernandez Cornejo & Ruiz Luna 2000).

The digestive gland is generally regarded as a major storage organ in decapods crustaceans (Allen 1971, Huggins & Munday 1968). The study of the digestive gland is of considerable interest because of its role in the accumulation and cyclic mobilization of reserves during the molting process, its contribution of nutrients to the ovary during vitellogenesis, the mobilization of its reserves during starvation, and its role in digestion and absorption. The level of the digestive enzymes in decapod crustaceans does not remain constant during the developmental cycles (Van Wormhoudt 1974) as a result of both external and internal factors. Among the external factors, the quantitative and qualitative variability of food is poignant.

Physiologic and biochemical effects of starvation have been

studied in several decapod species (Anger 1986, Cuzon et al. 1980, Cuzon & Ceccaldi 1973, Dall & Smith 1986, Wehrmann 1991), but little is known about the effects of prolonged food deprivation. Changes in biochemical composition during starvation have been reported in *Marsupenaeus japonicus* where there was a progressive suppression of metabolism compared with normally fed shrimp (Cuzon et al. 1980). The study found that the shrimp primarily used carbohydrates, then lipids to meet their energy requirement; proteins were significantly used only during the fourth week of starvation (Cuzon et al. 1980). In *L. vannamei* postlarvae, triacylglycerol provided energy during short periods of starvation whereas protein was used during prolonged starvation (Stuck et al. 1996).

Modifications in digestive enzyme activity have been found in several penaeid shrimp and related to the amount and quality of food (Lemos & Rodriguez 1998, Le Moullac et al. 1996, Le-Vay et al. 1993, Rodriguez et al. 1994). Rosas et al. (1995) found that the type and concentration of food influenced the ingestion rate of larval *L. setiferus*, which, in turn, affected metabolic rate. Changes in digestive enzyme activity were found under starvation conditions in juveniles of *Marsupenaeus japonicus* (Cuzon et al. 1980) and *Metapenaeus ensis* (Leung et al. 1990). Both studies found a decrease in digestive enzyme activity compared with fed shrimp.

Nitrogen excretion and metabolic rates are influenced by many factors, such as molt stage, feeding conditions, and level of activity. In this sense, O:N ratio has widely been used as an index of used substrate for oxidative metabolism (Chu and Ovsianico-Koulikowsky 1994, Dall & Smith 1986, Regnault 1981, Rosas et al. 1995). The catabolism of pure protein produced theoretical

*Corresponding author. E-mail: lcomoglio@hotmail.com

values of O:N of between 3 to 16 whereas the catabolism of equal quantities of proteins and lipids yield O:N values of between 50 and 60. Greater values of O:N correspond to an increase in lipid and carbohydrate catabolism (Mayzaud & Conover 1988).

The aim of this study was to evaluate the effect of starvation on digestive enzyme activity, oxygen consumption, ammonia excretion, and O:N ratio in *L. vannamei* juveniles. Afterwards, the effect of refeeding of starved juveniles was analyzed to assess the capability of shrimp to recover after prolonged starvation.

This information may contribute to a better understanding of the physiology of this commercially important shrimp species and will have an application in assisting with management in farms.

MATERIALS AND METHODS

Experimental Conditions and Design

Juveniles of *L. vannamei* were provided by a commercial farm, located in Mazatlán, Sinaloa, Mexico, during March 2001. The organisms were transported to the laboratory culture system of the Centro de Investigación en Alimentación y Desarrollo (CIAD) and fed with a commercial food during the acclimatizing period in 1000-L tanks. They were then transported to the laboratory, and groups of 20 organisms (wet weight = 0.998 ± 0.213 g) were maintained for different starvation periods (0, control group; 3, 6, 9, 12, and 15 days) in 10-L glass aquaria. Experimental conditions were 34 g · kg⁻¹ salinity, 24 °C water temperature, and 12D:12L. Water was completely renewed daily, and molts and dead organisms were removed. The natural seawater used in this study was pumped from a nearby beach (Cerritos beach), filtered through a cartridge filter of 5 µm and ultraviolet (UV) (Aquaplus, Mexico), and kept with aeration in a 600-L dark tank until use. Samples for bacterial load were regularly taken to confirm the absence of total heterotrophs and potential vibrios in the water.

Oxygen Consumption, Ammonia Excretion, and Atomic Ratio (O:N)

After each starvation period, groups of five individuals were placed in individual 500-mL respirometer chambers in a flow-through system using a continuous pump flow (ISMATEC, 12 mL/min flow). All measurements were done at the same time of day to obtain comparable data and assuming that all experimental shrimp were in minimum locomotive activity. Organisms were acclimated for 2 h in the beakers, and a sample of water from each chamber was taken to determine the initial concentration of oxygen and ammonia; flasks were sealed for 30 min, after which new samples were taken to measure final concentrations. The flow-through and the sealed periods were adjusted to avoid a depletion of oxygen concentration by more than 0.5 mg/L. Samples for ammonia excretion were filtered and fixed with H₂SO₄ (pH 2) and then frozen until processing.

The concentration of oxygen was measured using a polarographic oxygen electrode (YSI 59), and ammonia was determined by the indophenol technique (Parsons et al. 1984). Consumed oxygen and excreted ammonia were taken as being the net difference between the start and end of the sealed period. One out of every six chambers was left without a shrimp and measured as a control. The atomic O:N ratio was estimated according to Taboada et al. (1998) using the individual values of oxygen consumption and ammonia excretion transformed to µg At · g⁻¹ · h⁻¹ as follows: oxygen values were multiplied by 62.5 (1000 to convert the milligrams into micrograms divided by the atomic weight of oxygen, 16),

ammonia values were multiplied by 58.9 [obtained through dividing 1000 by the product of the atomic weight of nitrogen (14) and the fraction of nitrogen in NH₃ (0.824)].

Digestive Enzyme Activities

After physiologic measurements, shrimps were dissected and the hepatopancreas stored at -70 °C in individual 1.5-mL microtubes until enzyme assays were done.

Frozen samples were homogenized in 1 mL ice-cold pure water. Homogenates were centrifuged (at 14,000 × g for 6 min at 4 °C) and the aqueous supernatant, crude or diluted (1:10 v/v), was immediately used for enzyme analysis. The soluble-protein content was measured by the method of Bradford (1976), using a microplate reader at 495 nm. Duplicate assays for each sample were made. Trypsin activity was measured by the method of Erlanger et al. (1961) with N-α-benzoyl-DL-Arg-p-nitroanilide (BAPNA) as substrate. Chymotrypsin activity was assayed by the method of Delmer et al. (1979) using N-α-succinyl-L-alanyl-L-prolyl-L-phenyl-alanine-4-nitroanilide (SAPNA) as substrate. Hydrolysis for both enzyme activities was made in 0.1 M Tris-buffer, pH 8 at 25 °C, and the absorbance was measured at 405 nm. One unit of enzyme activity was defined as 1 µmol of p-nitroanilide liberated in 1 min at 25 °C.

α-amylase activity was assayed according to Bernfeld (1955) with 1.5% oyster glycogen as substrate in 10 mM phosphate buffer, pH 7. Absorbance measurements were made at 520 nm. For this method, one unit of enzymatic activity was defined as 1 mg of maltose liberated in 1 min at 37 °C.

General protease activity was estimated in homogenates using azocoll as substrate in phosphate buffer, pH 7.5 (Todd 1949). Absorbance was measured in a spectrophotometer at 520 nm. For this method, one unit was defined as the amount of enzyme that catalyzes the release of azo dye causing a $\Delta A/\Delta t = 0.001$ min (Walter 1988).

α-glycosidase activity was estimated using p-nitrophenyl-α-D-glycopyranoside as substrate in 50 mM phosphate buffer, pH 6. Absorbance was measured at 410 nm. One unit of enzymatic activity was defined as the amount of enzyme that hydrolyzes 1 µm of substrate per minute.

Post Starvation Refeeding Index

Other subgroups of organisms for each treatment (*n* = 6) were kept in individual chambers and exposed to a known amount of food previously lyophilized. The proximate composition of the food was 35% protein, 3.5% oil, 30% fiber, 16% ash, 12% humidity; average weight of each lyophilized pellet was 15.14 ± 2.28 mg; calorific content, 5.5 cal/mg of food. After 2 h 30 min, the nonconsumed food was removed and lyophilized again. The consumed food was calculated to measure the poststarvation refeeding index, defined as PSRFI (food consumed weight/body weight).

Bioenergetics Model

From a bioenergetic point of view, to integrate a model of energy partition for starved shrimp, we followed an equation (Bureau et al. 2000):

$$\text{DE requirement} = [\text{RE} + \text{Hem} + \text{HiE} + \text{HxE} \text{ [urine excretion energy (UE) + gill excretion energy (ZE)]} + \text{SE}]$$

This model originally included parameters where DE is digestible energy requirement, RE is energy gain, Hem is maintenance of energy requirement, HiE is heat increment of feeding, HxE is

nonfecal energy losses, and SE is surface loss or exuvia. With this model, a general energy balance model for starvation conditions in shrimp can be calculated.

However, in starved shrimps, the following parameters can be considered as 0:

$$DE = 0 \text{ (no feeding)}$$

$$HiE = 0 \text{ (no heat increment of feeding)}$$

$$SE = 0 \text{ (no exuvia)}$$

Hence, a model for starving shrimp could be

$$RE = -[Hem + HxE(UE + ZE)]$$

in which RE can be understood as endogenous energy needed to survive (Rosas, pers. comm.) and it is negative because in absence of energy input it is not possible to have energy gain *sensu stricto*. It is called also the scope for growth (SFG). Using this model, we calculated the endogenous energy (RE) for starving shrimp.

Statistical Analysis

To determine significant differences among starvation periods, one-way analysis of variance (ANOVA) and Tukey range test were used when the data were normal. Those results that were not normally distributed were evaluated through the Kruskal-Wallis test and Dunn's multiple comparisons (Daniels 1978). For both analyses, *P* was set at 0.05.

RESULTS

Weight and Survival

No significant differences were observed (*P* > 0.05) in relation to individual wet weight among treatments. However, survival decreased the longer the period of starvation (Table 1). The percentage of total molts were approximately 10% for 3- and 6-day treatments. No molting occurred after 6 days of starvation.

Oxygen Consumption, Ammonia Excretion, and O:N Ratio

Oxygen consumption increased significantly at 3, 6, and 9 days in starved animals (mean value $1.75 \text{ mg O}_2 \text{ h}^{-1} \cdot \text{g}^{-1}$) compared with the control. At 12 and 15 days, values were not significantly different from the control group (mean value $1.13 \text{ mg O}_2 \text{ h}^{-1} \cdot \text{g}^{-1}$) (*H* = 14.1; *P* = 0.015) (Fig. 1).

There were no significant differences in the amount of ammonia excreted between starved and control animals (mean value $0.08 \text{ mg N-NH}_3 \text{ h}^{-1} \cdot \text{g}^{-1}$, *H* = 1.74, *P* = 0.88) (Fig. 2). This may be because high standard deviations were observed, especially in the 6- and 12-day starvation groups (0.03 and $0.067 \text{ mg N-NH}_3 \text{ h}^{-1} \cdot \text{g}^{-1}$, respectively).

No significant differences between treatments were observed

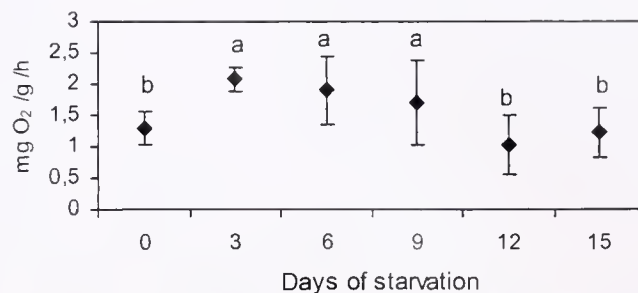


Figure 1. Routine oxygen consumption of juveniles of *L. vannamei* after each starvation period (mean \pm SD). Treatments sharing the same letters are not significantly different (*P* > 0.05).

with regard to O:N atomic ratios (*H* = 10.14, *P* = 0.071) (Fig. 3). However, there was a trend toward increased ratios between days 3 and 9, suggesting that lipid was being catabolized. By day 12, the drop in the ratio to 10–20 suggests that protein was once again the main source of energetic fuel.

Enzyme Activity

Significant differences were found in the hepatosomatic index after different periods of starvation (*H* = 25.6, *P* = 0.0001). The index was significantly lower after animals were starved beyond 9 days. Beyond the ninth day, index decreased a further 50% until reaching the end of the experiment (Table 1). Hepatopancreatic total soluble protein was significantly affected by the period of starvation (ANOVA, *P* < 0.05). The highest value ($268.7 \pm 52.13 \text{ mg/mL}$) was obtained in the control, followed by the 3- and 6-day starvation experiments (mean value 159.8 mg/mL). The lowest significant values were found in the 9- to 15-day experiments (53.23 mg/mL) (Fig. 4).

Digestive enzyme activity was, in general, significantly affected by different periods of starvation. Hepatopancreatic digestive carbohydrases results are shown in Figure 5. Total amylase activity was significantly decreased by starvation (ANOVA, *P* < 0.05), from the highest value in fed juveniles (control) ($0.016 \pm 0.002 \text{ total U}$), followed by the 3- and 6-day experiments (mean value 0.011 total U) and the lowest value in the 9-, 12-, and 15-day starved groups (mean value 0.007 total U) (Fig. 5a). However, the specific activity increased as the soluble protein content decreased (Fig. 5b).

Glycosidase activity was significantly decreased (mean value 1.47 total U) by all starvation periods as compared with control fed animals ($3.6 \pm 1.5 \text{ total U}$) (Fig. 5c). For this enzyme, the specific activity also increased as the soluble protein content in the hepatopancreas decreased (Fig. 5d).

Proteinase activity was significantly affected by the starvation

TABLE 1.
Wet weight (g), survival (%), and hepatosomatic index (%) of the food-deprived shrimps *L. vannamei*.

	Starvation Days					
	0	3	6	9	12	15
Wet weight (g)	1.07 ± 0.1	0.86 ± 0.2	0.97 ± 0.23	0.99 ± 0.3	0.85 ± 0.1	1.04 ± 0.12
Survival (%)	100	90	90	65	65	55
Hepatosomatic index (%)	4.1 ± 0.7	3.3 ± 0.4	2.90 ± 0.45	2.5 ± 0.4	2.1 ± 0.3	1.98 ± 0.9

Data are mean \pm SD.

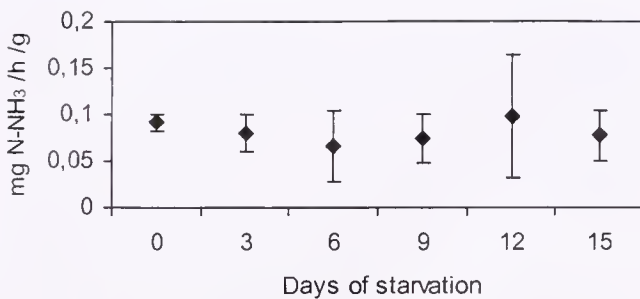


Figure 2. Routine nitrogen excretion of juveniles of *L. vannamei* after different starvation periods (mean \pm SD).

periods (ANOVA, $P < 0.05$) (Fig. 6). The highest value (5.3 ± 2.4 total U) was observed in the control, which was not significantly different from 3- and 6-day experiments, whereas the lowest value of total proteinase activity (1 ± 0.12 total U) was observed in the shrimps starved for 15 days (Fig. 6a). As with the carbohydrases, the specific activity of total proteinases was highest in shrimps starved for 12 days (Fig. 6b).

Total trypsin activity was significantly lower from the ninth day to the end of starvation (mean value 0.63 total U). Fed shrimp and those deprived for 3 and 6 days yielded a mean trypsin activity value of 2.58 total U (Fig. 6c).

Total chymotrypsin activity decreased over the period of starvation. Significant differences were found among the fed shrimps (45.41 ± 11.89 total U), the 3- and 6-day starvation groups (mean value 25.02 total U), and the 9-, 12-, and 15-day starvation groups (mean value 7.87 total U) (Fig. 6e). The specific activities of these latter two endoproteinases were not significantly affected by starvation (Figs. 6d and 6f).

Poststarvation Refeeding Index

The PSRFI was not significantly different between the control and 3- and 6-day starvation experiments (PSRFI = 5.09%, 4.71%, and 4.75%, respectively) whereas the amount of food consumed decreased for the 9-, 12-, and 15-day starvation experiments (PSRFI = 3.72%, 3.3%, and 2.97%, respectively) (Fig. 7) (Kruskal-Wallis, $H = 21.5$; $P = 0.006$).

Bioenergetics Model

Estimation of RE values indicates that at the beginning as well as at the end of the starvation period, shrimp needed the same endogenous energy to survive, whereas between 3 and 9 days they needed more energy, used essentially in respiration to maintain

basal metabolism (Table 2). However, the routine respiration rate measured was 61% higher at day 3 of starvation than in fed shrimp and remained at this high level until day 9 of starvation. After then, values decreased to the same value as in controls.

DISCUSSION

In the current study the wet weight did not change with starvation, but survival rate decreased, and the suppression of molt after 6 days of starvation was observed. Anger and Spindler (1987) have observed in *Hyas araneus* a delay in molt under starved conditions and associated it with the point of reserve saturation (PRS). The PRS is the critical point where sufficient energy and/or essential substances have been accumulated to allow autonomous (food-independent) development through the entire rest of the molt cycle. Stuck et al. (1996) have also observed the inhibition of molting in response to starvation in *L. vannamei*. Under starvation conditions, a first event commonly observed is a weight loss in relation to energy expenditure for basal metabolism; after a few days, shrimp in premolt stages will not evolve further and refrain from exuviation, saving around 1.4 kJ (Read & Caulton 1980), which is the energy expenditure at molt. This means that shrimp have the adaptation to tolerate starvation, saving energy from exuvia, including the energy challenged to mobilize reserves, chitin digestion, and exuviation. In this way, shrimp could compensate for weight loss, maintaining body weight without significant changes, as was observed in the current study.

Litopenaeus vannamei juveniles, as many species of crustaceans, showed a biochemical adaptation response to an absence of food (decrease of digestive enzyme activities) using their own reserves (hepatopancreatic glycogen, protein, and probably lipids, estimated through O:N ratio variations) for homeostasis and to channel enough energy for basal metabolism in that period (Cuzon and Ceccaldi 1973, Dall and Smith 1986; Dall and Smith 1987, Leung et al. 1990).

Homeostasis in shrimp can change to help animals sustain severe food deprivation and survive; it is not instantaneous as the poststarvation and refeeding showed; and not all starved shrimps stayed alive (Table 1). It seems that under the experimental conditions tested, there was a kind of discrimination between shrimps that were capable of surviving starvation and those that were not. Some individuals may be more able than others to change some metabolic routes and resist food deprivation without significant morbidity. The previous life history of the animal before animals were sampled in the farm and used in experiments may also influence the variability between individuals to survive starvation. It

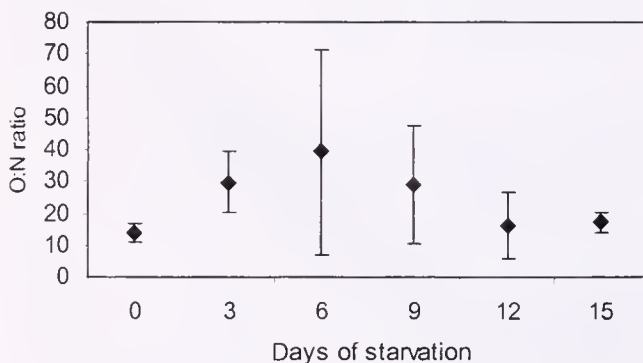


Figure 3. O:N ratio of food-deprived juveniles of *L. vannamei*.

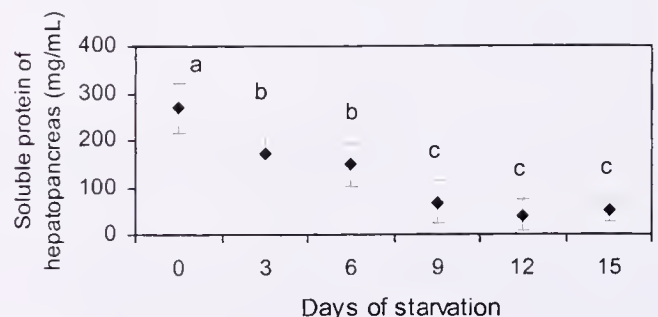


Figure 4. Hepatopancreatic total protein of the food-deprived juveniles of *L. vannamei* (mean \pm SD). Treatments sharing the same letters are not significantly different ($P > 0.05$).

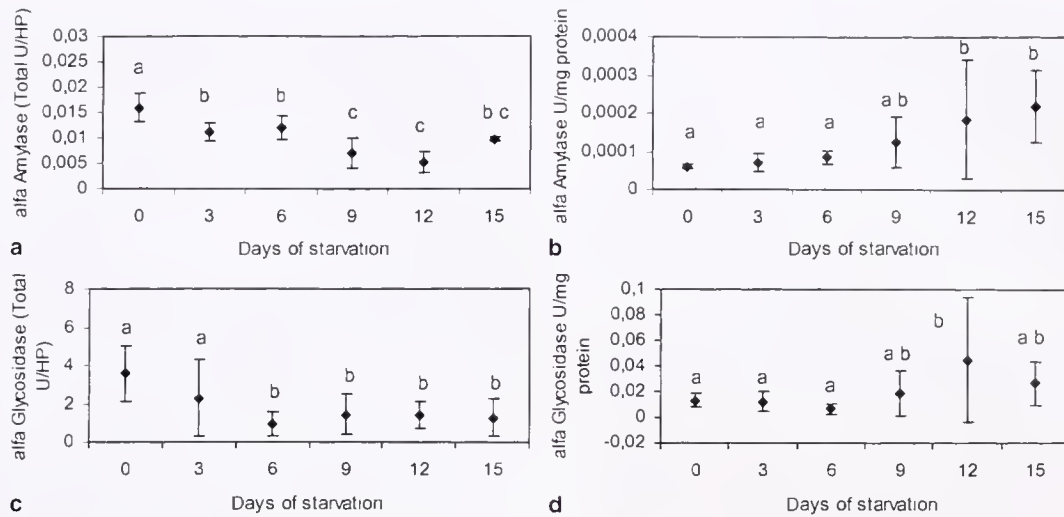


Figure 5. Total and specific activities of α -amylase (A and B) and α -glycosidase (C and D) of food-deprived juveniles of *L. vannamei* (mean \pm SD). Treatments sharing the same letters are not significantly different ($P > 0.05$).

must also be appreciated that these results will be biased due to the selection by farmers of the most robust and fastest growing individuals in the population.

According to these results, a high rate of mobilization of reserves was observed between days 3 and 9 of starvation, when the recovered energy (RE) was maximal. Shrimp reserves are mainly limited to lipids stored in the digestive gland. When shrimp are starving, they will use those reserves, increasing the energy debt and reducing the digestive gland weight and its components. Reduction of enzyme activity and soluble protein during starvation

underlined the change. It means the shrimp are biochemically and energetically well adapted to fasting because they could mobilize their reserves to be used as energetic sources; at the same time, the enzyme activity in the digestive gland was maintained. This type of strategy has been observed in other shrimp species. Cuzon et al. (1980) showed that *M. japonicus* used protein to obtain energy though the catabolism of amino acids present in digestive gland cells during a prolonged starvation period. A similar response was observed in *Penaeus esculentus* by Smith and Dall (1991), evidencing that shrimp can mobilize their own energetic reserves

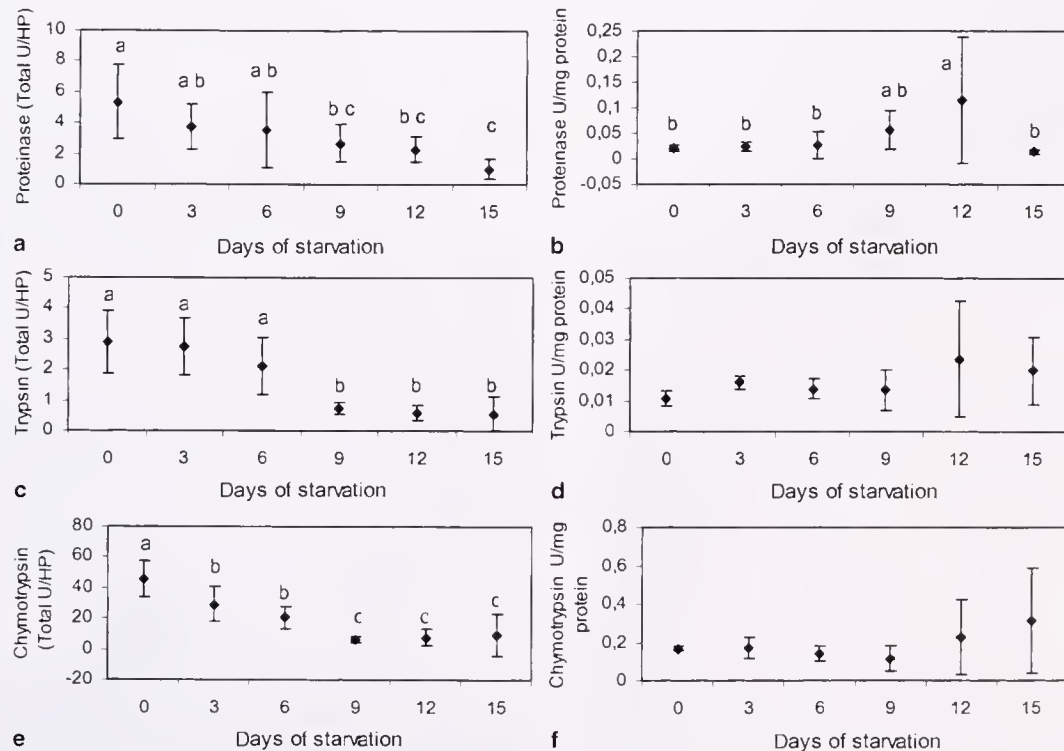


Figure 6. Total and specific digestive proteinases activities of juveniles of *L. vannamei* after different starvation periods (mean \pm SD). Treatments sharing the same letters are not significantly different ($P > 0.05$).

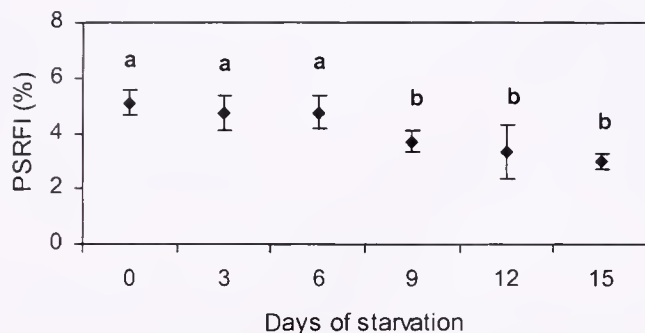


Figure 7. Ingestion rate of juveniles of *L. vannamei* after the different starvation periods (PSRFI %) (mean \pm SD). Treatments sharing the same letters are not significantly different ($P > 0.05$).

through catabolizing lipids (after 3 to 6 days of starvation) or protein to sustain themselves through food deprivation. Otherwise, a negative correlation was observed in starvation condition between total hepatopancreatic soluble protein and total activity for all measured digestive enzymes. This correlates well with the lower values of poststarvation refeeding rate.

In general terms, digestive enzymes follow the presence or absence of food. Samain et al. (1983) found that amylase increases in case of food deprivation with a peak, and then the enzyme production decreases as an adaptation to low nutrition status and to save energy. Digestive enzymes of *M. japonicus* showed a similar trend that supports previous results (Cuzon et al. 1980). However, results obtained after refeeding indicate that the necessary time to recover the digestive gland integrity depends on the fasting period. These results show that although *L. vannamei* have an adaptation mechanism to tolerate fasting conditions, recovery cannot be achieved if starvation is long enough to produce physical damage in the digestive gland and loss of enzyme synthesis. A pattern of variation for digestive enzymes presented by *L. vannamei* during a shorter experimental period differed from the one of *M. japonicus*. At day 15, for example, specific enzymes activity increased as a sort of adaptation to absorb the minute amount of food, but as energy expenditure increased, the peak disappeared shortly. Both amylases and proteases exhibited the same trend.

Mayzaud and Conover (1988) described starvation condition regarding the use of energetic substrates estimated through O:N ratio. It can be assumed that acetyl CoA, which is the final product of β -oxidation of fatty acids, could be challenged into biologic

TABLE 2.

Energetic balance of starved juveniles of *L. vannamei* at different starvation days.

Starvation Days	Hem (J · d ⁻¹ · g ww ⁻¹)	HxE (J · d ⁻¹ · g ww ⁻¹)	RE (J · d ⁻¹ · g ww ⁻¹)
0	449 ± 94 ^b	53 ± 5 ^a	-473 ± 77 ^b
3	725 ± 68 ^a	46 ± 12 ^a	-771.7 ± 65 ^a
6	664 ± 187 ^{ab}	39 ± 22 ^a	-696 ± 178 ^{ab}
9	598 ± 233 ^{ab}	43 ± 15 ^a	-655 ± 262 ^{ab}
12	361 ± 167 ^{bc}	57 ± 39 ^a	-418 ± 166 ^b
15	428 ± 138 ^b	45 ± 16 ^a	-473 ± 152 ^b

Hem, maintenance of energy requirement; HxE, nonfecal energy losses; RE, the endogenous energy; ww, wet weight. Data are mean \pm SD.

^{a,b,c} Values in the same column that share the same superscript letter do not differ significantly ($P > 0.05$).

TABLE 3.

Theoretical limits of the O:N ratio according to Charmantier.*

Days of Fasting	J ₁	J ₃	J ₆	J ₁₀	J ₁₅
O:N	70–100		50–60		2–16
Substrate	Glycogen		Triglycerides		Amino acids

* Charmantier (pers. comm.).

oxidation, requiring 46–52 oxygen atoms whereas, in normal condition, β -oxidation of neutral lipids requires 14–16 oxygen atoms to produce acetyl CoA. Increase in O₂ consumption of shrimps between days 3 and 9 of starvation with no increase of nitrogen excretion resulted in a high O:N ratio. Then, β -oxidation of hepatopancreatic lipid reserves could be occurring in this period. An explanation for a change can be found in the O:N ratio in which the use of lipid as energetic substrate was clear followed by protein as the main energy source. Ammonia excretion values provided indication on substrate oxidation; although nitrogen excretion was not significantly different from the control along the starvation period, O:N ratio values showed a trend to use lipids as energetic substrate between days 3 and 9 of starvation. Such a trend has also been evidenced in *M. rosenbergii* (Clifford & Brick 1983). After 12 starvation days, shrimp returned to the use of protein to derive energy. Likewise, as Mayzaud and Conover (1988) reported for planktonic crustaceans, a decrease in O:N ratio with time of starvation seemed to be common to all species with a predominantly protein-based metabolism. O:N ratio variation can be related to glucose homeostasis, through the regulation of glycconeogenesis (Cuzon et al. 2001) and glycolysis (Hochachka et al. 1988). These results are not in contradiction to those reported by Dall and Smith (1986) for *Penaeus esculentus*. These authors pointed out a reduction in metabolic rate with starvation providing a mechanism for prolonged survival. O:N ratios gave an explanation for successive fuel substrates as starvation increased in intensity (Table 3). Glycogen in the hepatopancreas is affected first, as in *Crangon crangon* (Regnault 1972), then neutral lipids are hydrolyzed, and toward the end of the starvation period, protein is used similarly as in *M. japonicus* juveniles (Cuzon et al. 1980).

This study has shown that *L. vannamei* is unable to sustain food deprivation for periods longer than 15 days. By comparison, the temperate species *M. japonicus* can survive 4 weeks of starvation. Both species show similar levels of digestive enzyme activity. However, basal metabolic rates in the former are higher because of its tropical habitat (24–27°C) as opposed to the temperate conditions (20°C) inhabited by the latter. They possess a similar level of digestive enzyme activities; then, there are good reasons, according to values of metabolism rates obtained in this study, to think that tropical species present a higher basal metabolism and activity that leaves them more dependent on regular food supply for their development.

The current work indicates *L. vannamei* is dependent on a regular food supply for its development, which has implications for its management in farms.

ACKNOWLEDGMENTS

This study was funded by CONACYT Project No. 34952-b (A. Roque), UNAM-DGAPA-IN-231599 (G. Gaxiola), CONICET-Argentina (L. Comoglio and O. Amin), and IOC (O. Amin). The authors thank Dr. Carlos Rosas for critically reviewing the manuscript and Mrs. Ingrid Mascher for editorial assistance.

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EFFECT OF DIETARY ASCORBIC ACID LEVELS ON REPRODUCTIVE PERFORMANCE OF SHRIMP, *LITOPENAEUS VANNAMEI* (BOONE), BROODSTOCK

SHAOBO DU, CHAOQUN HU AND QI SHEN

Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, The Chinese Academy of Sciences, 510301, Guangzhou, People's Republic of China

ABSTRACT This study was conducted to investigate the effect of dietary ascorbic acid (AA) on reproductive performance of shrimp *Litopenaeus vannamei*. Stay C-35 (a stable derivative, mainly monophosphate) was selected as AA source. Four grade levels of ascorbic acid equivalent (AAE) (0, 400, 800, and 1200 mg kg⁻¹ diet) were supplemented to the basal diet. Gonadosomatic index (GSI), hepatosomatic index (HSI), fecundity, and egg diameter were not significantly affected by supplemented AA levels ($P > 0.05$), although AA concentrations in hepatopancreas, ovaries, and eggs increased significantly with dietary AA levels ($P < 0.05$). Average daily spawns per female, hatching rate, and fertilization rate were also significantly affected by dietary AA levels ($P < 0.05$). Results of this study confirmed the importance of supplementation of AA to the basal diet and suggested that at least 800 mg AAE kg⁻¹ diet was needed to supplement to the basal diet to acquire excellent ovarian maturation and reproductive performance.

KEY WORDS: ascorbic acid, broodstock, *Litopenaeus vannamei*, reproductive performance

INTRODUCTION

Although nutrition has been identified as a key factor in the reproduction of broodstock shrimp, information on broodstock nutrition is still limited (Harrison 1990, Wouters et al. 2001b). Studies into the effect of nutrients on reproduction are essential to formulate high-quality broodstock diets as well as to elevate reproductive performance and quality of hatched nauplii.

Dietary ascorbic acid (AA) is an essential nutrient for penaeid shrimp (He & Lawrence 1993). Available studies into the role of this vitamin have focused on its effect in immature stages and suggested a dietary source of AA is required to prevent deficiency symptoms, such as reduced growth, melanized lesions underneath the exoskeleton, poor wound repair, and high mortality (Hunter et al. 1979, He & Lawrence 1993, Magarelli et al. 1978, Shiao & Hsu 1994). The importance of AA in reproduction has also been reported in fish and shrimp. In addition to the role of AA as an antioxidant and an enzyme cofactor in the formation of collagen (Barnes & Kodicek 1972, Hunter et al. 1979), which may be especially important during embryonic and larval development, it is also involved in the regulation of biosynthesis of steroid hormones (Hilton et al. 1979, Levine & Morita 1985, Seymour 1981). The essentiality of AA for fish broodstock was well established (Lavens et al. 1999a, Mangor-Jensen & Holm 1994, Sandnes et al. 1984, Soliman et al. 1986, Waagbo et al. 1989), and the importance of AA in shrimp reproduction has also been confirmed. Alava et al. (1993a, 1993b) reported AA-deficient diet retarded gonadal maturation of *Marsupenaeus japonicus*. Cahu et al. (1995) found AA levels in *Farfantepenaeus indicus* eggs were affected by dietary vitamin levels, and high hatching rate of *F. indicus* eggs were related to high AA levels in the eggs. Wouters et al. (2001a) detected a sharp decrease of AA content in spent ovaries and nauplii of *Litopenaeus vannamei* and suggested that AA was consumed during egg development and hatching.

Till now, there is still no information on the effect of dietary AA on ovary maturation and reproduction of *L. vannamei*. In a previous study, we successively substituted a natural diet consisting of 50% bloodworm (*Glycera chironi*) and 50% oyster (*Crassostrea rivularis*) with an artificial diet for broodstock *L. vannamei*. In the current study, we used this artificial diet as the basal diet and selected Stay C-35, a stable derivative, as AA source to investigate the effect of dietary AA on the reproductive perfor-

mance of *L. vannamei*. The effect of dietary AA levels on AA concentrations in ovaries, hepatopancreas, and eggs was also detected.

MATERIALS AND METHODS

Animals and Treatments

Domestic *L. vannamei* breeders were obtained from Dongfang Co. (Zhanjiang, China). They were kept in maturation tanks for 3 wk to acclimate experimental conditions and were fed the basal diet without any AA supplemented (Diet C1). A unisex system was used as described by Browdy et al. (1996): 4 tanks were stocked with 15 females each and 4 tanks with 15 males each. They were divided into four groups: Group C1–C4, which were subjected to Diet C1–C4, respectively. Each group consisted of a male tank and a female tank. After acclimation, female shrimps were unilaterally eyestalk-ablated with a pair of burned tweezers. To identify each female in the same group, part of its telson was cut, except five females were left intact and later sampled to test gonadosomatic index (GSI) and hepatosomatic index (HSI). The postablation phase of the experiment lasted for 50 days.

The maturation tanks were rectangular-shaped cement tanks (2 × 3 m², 55 cm water depth) in which sand-filtered and UV-treated seawater was exchanged at a rate of 200% daily. The physicochemical parameters of the water were average temperature, 28.5 °C; average salinity, 30 mg L⁻¹; photoperiod, 12 h light/12 h dark; and light intensity, 20 mW m⁻². Under these conditions, oxygen remained close to saturation, 6.2 g m⁻³, pH was 8.2 ± 0.1, while ammonia, nitrite, and nitrate levels remained almost the same as in the inlet water.

Diets and Feeding

In a previous study, the effect of 100% fresh food replacement with an artificial diet on ovarian maturation and spawning performance of broodstock *L. vannamei* was tested. Results showed that 100% artificial diet gave superior spawning performance to the fresh food control (consisting of 50% *G. chironi* and 50% *C. rivularis*), such as higher total spawn numbers (32 vs. 24), maturation ratio (90% vs. 70%), and daily spawns per female (0.091 vs. 0.069). As such, a fresh food replacement level of 100% was selected for the current study, and the same basal diet—with the

following composition related to dry matter: protein 48.98%, lipid 11.57%, and ash 15.42%—was used in the experiment. Ingredients and composition of basal diet are shown in Table 1. Four grade levels of ascorbic acid equivalent (AAE) were added to the basal diet (Table 2). ROVIMIX STAY C-35 (mainly monophosphate, containing 350 g L-AA kg⁻¹) was selected as the AA source for its excellent stability and bioavailability (Alexis et al. 1999). The diets were prepared in the following manner. All dried ingredients were smashed and sieved, mixed with 35% (w/w) water, and the resulting dough was pelleted with a meat grinder and dried at room temperature. Then the dried “spaghetti-like” 3-mm-diameter strands were crumbled to about 5-mm long and kept in sealed plastic bags at -20°C until use. Shrimps were fed 4 times daily (0800, 1100, 1400, and 1800 local time) with a measured ratio of 6% of the tank biomass (wet weight). Uneaten food was collected daily.

Broodstock Maturation and Spawning

After unilateral ablation, the females were visually examined for ovarian maturation stages at time 2000 every night according to Wouters et al. (2001a). For the females with intact telsons, when the ovary of the first female developed to stage VI, they were weighed and dissected, while keeping them on ice, to determine the gonadosomatic index (GSI = 100 × gonad weight/total body weight) and hepatosomatic index (HSI = 100 × hepatopancreas weight/total body weight).

The remained females ready to spawn were transferred to the corresponding male tanks to mate. Mature females with an attached spermatophore were placed in individual 120-L spawning tanks and after spawning were returned to their own maturation tanks. Fecundity (number of eggs per spawn) was estimated by

TABLE 1.

Ingredients and proximate biochemical composition of the basal diet.

Ingredients	Weight (g kg ⁻¹ diet)
Fish meal	340
Oyster (<i>Crassostrea rivularis</i>) meal	200
Bloodworm (<i>Glycera chirori</i>) meal	100
Alpha starch	206.57
Shrimp head meal	50
Alaska fish oil	40
Soybean lecithin	15
Ca(H ₂ PO ₃) ₂ · H ₂ O	10
Cholesterol	5
Sodium alginate	20
Vitamin mix*	10
Proximate biochemical composition	Percentage (%)
Moisture	5.91 ± 0.25
Ash†	15.42 ± 0.11
Crude protein†	48.98 ± 0.69
Total lipid†	11.57 ± 0.84

* Vitamin composition (mg/IU kg⁻¹ diet): vitamin A palmitate, 100,000 IU; vitamin D₃, 10,000 IU; biotin, 7 mg; α-tocopherol acetate, 350 mg; meso-inositol, 800 mg; nicotinine, 300 mg; Ca-pantothenate, 600 mg; pyridoxine HCl, 100 mg; riboflavin, 120 mg; thiamin HCl, 50 mg; folic acid, 13 mg; cyanocobalamin, 1 mg; menadione bisulfite, 60 mg; choline chloride 2000 mg.

† Ash, protein, and lipid content was calculated based on dry matter.

TABLE 2.

Supplemented ascorbic acid levels of the experimental diets.

Variable Ingredients	C1	C2	C3	C4
Carboxymethylcellulose (mg kg ⁻¹ diet)	3428.57	2285.71	1142.86	0
Stay C-35 (mg kg ⁻¹ diet)*	0	1142.86	2285.71	3428.57
AAE (mg kg ⁻¹ diet)	0	400	800	1200

AAE, ascorbic acid equivalent.

* ROVIMIX Stay C-35 (Roche Sunve Vitamins Ltd., Shanghai), mainly monophosphate, containing 350 mg L-ascorbic acid g⁻¹.

stirring the spawning tanks and counting three subsamples of 50-mL each. Fertilization rate was assessed in three 50-mL samples of each spawn, based on the presence of a double membrane in the eggs. A sample of 10,000 eggs was incubated at 29°C for estimating the hatching rate (% nauplii/fertilized eggs). All spawns were hatched individually. Hatching rate was calculated by counting the number of nauplii per spawn after positive phototropism selection. Egg diameter was estimated with a light microscope and a micrometer. About 80 mg eggs were sieved from each spawn and immediately rinsed in freshwater and stored at -70°C. Egg samples from the same group were pooled to obtain sufficient eggs for AA analysis. At the end of the experiment, five females with ovaries of stage II from each group were dissected. Hepatopancreas and ovaries were pooled and stored at -70°C for latter AA analysis.

Biochemical Analysis

Triplicate biochemical analysis of the basal diet was conducted according to the following standard procedures (AOAC 1990). Moisture was determined by oven drying to constant weight at 105°C. Crude protein (N × 6.25) was derived from Kjeldahl nitrogen analysis. Ash was determined at the residue after muffle furnace ignition at 550°C for 6 h. Total lipid content was determined by Soxhlet extraction with petroleum ether at 60°C for 8 h.

Tissue AA determinations were conducted by HPLC (HP1100) according to Alava et al. (1993b) and modified slightly. The analytical condition of HPLC was as follows: detection at UV-240 nm; column temperature, 30°C; eluent, 0.1 M KH₂PO₄ + 0.5% metaphosphoric acid w/v, pH 3.4; flow speed, 0.8 mL/min. AA was extracted from samples by the method of Nelis et al. (1997). Freeze-dried samples of 0.5 g were suspended in the extractant of 1% acetic acid-0.1% metaphosphoric acid-1 mM EDTA and ultrasonically homogenized for 10 min in an ice bath. The mixture was centrifuged for 5 min at 12,000g and passed through a 0.45-μm cellulose acetate membrane filter (SIGMA). Ten microliters of filtrate was introduced in the HPLC injection port. AA (Biochemika ultra, SIGMA) was used as standard.

Statistical Analyses

Each female in the same group was considered as an experimental unit for replication. Data of GSI, HSI, fecundity, daily spawns per female, egg diameter, fertilization rate, and hatching rate from each group were subjected to one-way ANOVA and subsequent Duncan's multiple-range test to determine difference in means. Prior to analysis, Levene's test for homogeneity of variances was used to verify the assumptions for further analysis.

There was no need to transform data. A regression analysis was used to determine the relationship between fecundity, egg diameter, fertilization rate, and hatching rate and spawn order. No correlations were detected between them, and spawn order was not considered as an additional factor to evaluate. An alpha level for all tests was set at 0.05. Statistical analysis was performed using Systat package (SYSTAT, 1996).

RESULTS

Average initial weight of female and male shrimps was 49.44 ± 4.29 g and 48.04 ± 4.81 g, respectively. As slightly mortality, probably induced by manipulation, occurred in some groups after 30 days, number of spawns was calculated per female per day.

Reproductive performance of different groups is shown in Table 3. Results showed that GSI increased with the increasing levels of AA, but the difference was not significant ($P > 0.05$). HSI values of female shrimps were also not significantly affected by dietary AA levels, though the HSI values of groups C2, C3, and C4 were slightly higher than that of group C1. Average daily spawns per female increased significantly with the AA levels ($P < 0.05$), and females of group C3 had the highest daily spawns, which was significantly higher than that of group C1 (0.074 vs. 0.050). Fecundity of all the females was similar, and there was no significant difference between the groups ($P > 0.05$). Similarly, egg diameter was almost the same and seemed not to be affected by dietary AA levels. Supplementation of AA to the basal diet increased the fertilization rate and hatching rate significantly ($P < 0.05$). Fertilization rate of group C2 (62.58%) was significantly higher than that of group C1 (53.76%) whereas group C3 had the highest fertilization rate (71.70%), which was significantly higher than those of groups C2 and C1. Hatching rate of group C4 was highest (61.08%), but was not significantly different with group C3 (58.13%), and the hatching rate of groups C1 (37.88%) and C2 (47.58%) was significantly lower than those of groups C3 and C4.

AA content in hepatopancreas, ovaries, and eggs of female *L. vannamei* is shown in Table 4. These females were at maturity stage II, with mean GSI values of 1.54 ± 0.05 for group C1, 1.57 ± 0.05 for group C2, 1.63 ± 0.09 for group C3, and 1.60 ± 0.06 for group C4, respectively. Results showed that there were much higher concentrations of AA in ovaries than in eggs and hepatopancreas. Tissue AA content was significantly affected by dietary AA levels. There were 19.34 mg kg^{-1} and 21.36 mg kg^{-1} in the hepatopancreas of groups C1 and C2, which were signifi-

cantly lower than those of groups C3 (37.13 mg kg^{-1}) and C4 (40.79 mg kg^{-1}). AA concentrations in ovaries and eggs increased significantly with the increasing AA levels ($P < 0.05$), and there was significant difference between the different groups.

DISCUSSION

The current study showed the importance of supplemental AA in broodstock diets of *L. vannamei* for enhanced ovarian maturation and reproduction. It appeared that AA concentration in the basal diet was sufficient to maintain normal survival, successive maturation, and spawns; however, for significantly higher daily spawns, fertilization rate, and hatching rate, supplementation of this vitamin to broodstock diet was necessary.

GSI and HSI indicated the effect of AA levels on the development of ovaries and hepatopancreas of female *L. vannamei*. GSI and HSI values of females fed the diet without AA supplementation were lower than those fed AA-supplemented diets, although the difference was not significant ($P > 0.05$). The period between eyestalk ablation and the first maturation of the females with intact telsons was only 5 days, and the relatively short period of time might account for the similar values of GSI and HSI. Females fed diets supplemented with 800 and 1200 mg kg^{-1} AAE gave significantly higher daily spawns than those fed nonsupplemented diet. This result indicated that supplementation of at least 800 mg kg^{-1} AAE to the basal diet was able to significantly enhance the ovarian maturation of female *L. vannamei*. Alava et al. (1993a, 1993b) also concluded the positive effect of AA on ovarian maturation of *M. japonicus* and suggested at least 500 mg kg^{-1} AMP in the broodstock diet was necessary for ovarian maturation.

In this study, AA concentrations in hepatopancreas, ovaries, and eggs were widely affected by dietary AA levels. AA content in ovaries and eggs increased significantly with the increasing dietary AA levels. The similar trend has been confirmed in the studies on *M. japonicus* conducted by Alava et al. (1993b) and that on *F. indicus* by Cahu et al. (1995). There were higher levels of AA in ovaries than in hepatopancreas, as was shown in *M. japonicus* (Alava et al. 1993b), *Palaemonetes pugio* (Coglianese & Neff 1981), and *Macrobrachium rosenbergii* (Cavalli et al. 2001). The ovaries sampled in the current study were from females at maturation stage II, so the high levels of AA in pre-mature ovaries might be related to an increased requirement in the eggs as suggested by Hilton et al. (1979). Sandnes et al. (1984) and Soliman et al. (1986) showed that broodstock dietary AA was transferred to

TABLE 3.
Reproductive performance of *L. vannamei* fed diets supplemented with different AA levels.

	C1	C2	C3	C4
Survival rate of males	93.33%	93.33%	86.67%	93.33%
Survival rate of females	90%	90%	100%	100%
GSI	1.38 ± 0.35	1.62 ± 0.28	1.75 ± 0.36	1.83 ± 0.74
HSI	3.19 ± 0.72	4.05 ± 0.42	3.62 ± 0.46	3.53 ± 0.82
Average spawns per female	2.5	3.3	3.7	3.5
Daily spawns per female*	0.051 ± 0.018^a	0.067 ± 0.018^{ab}	0.074 ± 0.019^b	0.070 ± 0.019^b
Fecundity ($\times 10^3$)	120.8 ± 29.4	126.9 ± 25.6	135.1 ± 29.0	128.6 ± 36.7
Egg diameter (μm)	268.61 ± 4.30	267.53 ± 2.64	268.90 ± 5.37	268.31 ± 3.52
Fertilization rate (%)	53.76 ± 10.27^a	62.58 ± 8.30^b	71.70 ± 9.21^c	68.44 ± 8.03^{bc}
Hatching rate (%)	37.88 ± 6.66^a	47.58 ± 9.11^b	58.13 ± 10.98^c	61.08 ± 11.11^c

GSI, gonadosomatic index; HSI, hepatosomatic index.

* Values in the same row with different superscripts were significantly different ($P < 0.05$).

TABLE 4.

AA concentrations (mg/kg) in hepatopancreas, ovaries, and eggs of *L. vannamei* fed diets supplemented with different AA levels.

	C1	C2	C3	C4
Hepatopancreas*	19.34 ± 0.18 ^a	21.36 ± 0.54 ^a	37.13 ± 0.52 ^b	40.79 ± 0.49 ^b
Ovary*	75.04 ± 0.31 ^a	100.00 ± 0.93 ^b	109.16 ± 0.71 ^c	115.06 ± 0.80 ^d
Egg*	47.68 ± 0.24 ^a	52.02 ± 0.76 ^b	61.59 ± 0.57 ^c	65.46 ± 1.10 ^d

AA, ascorbic acid.

* AA concentrations were based on dry weight. Values in the same row with different superscripts were significantly different ($P < 0.05$).

eggs where it was stored for use during embryogenesis and development of larvae until the first feed intake. The positive effect of AA in shrimp production might be associated with the general role as an antioxidant (Wouters et al. 1999) as well as its action in the hydroxylation of protein-bound proline and lysine, which provide stable triple helical collagen through the embryonic stages (Barnes & Kodicek 1972). Guary et al. (1975) postulated that ovarian AA could be consumed during steroidogenesis, and the study of Calvalli et al. (2001) provided evidence for the possible demand for AA by the hydroxylating reactions in steroidogenesis in the ovarian follicle cells. Similarly, Wouters et al. (2001a) detected higher AA concentrations in immature, maturing, and mature ovaries than in ovaries of spent females and nauplii of *L. vannamei*, and suggested that the lost AA was used during egg development and hatching.

Results of the current study showed that supplementation of more than 800 mg kg⁻¹ AAE to the basal diet could significantly increase the fertilization rate and hatching rate of broodstock *L. vannamei*. Cahn et al. (1995) also suggested a positive relationship between improvement of egg hatchability and high AA concentration in broodstock diets as well as in eggs of *F. indicus*. The same positive effect of supplemented AA on egg quality and hatching rate was also observed in fish (Sandnes et al. 1984, Soliman et al. 1986), and the feeding of an AA-deficient diet to broodstock fish could diminish reproductive performance (Hilton et al. 1979).

A dietary source of AA is required by all species of shrimp tested to date (Conklin 1997), though a possible limited synthesis

of AA was suggested in *P. californiensis* and *L. stylirostris* by Lightner et al. (1979). He & Lawrence (1993) indicated that dietary AA requirement of *L. vannamei* was size-dependent and decreased with increased size. They reported that minimum dietary AA levels required for normal survival were 120 mg kg⁻¹ and 41 mg kg⁻¹ diet for shrimp with an initial weight of 0.1 g and 0.5 g, respectively. Similarly, Lavens et al. (1999b) pointed out that for early postlarval *L. vannamei*, an optimal dietary AA level of 130 mg kg⁻¹ diet was needed to acquire best growth performance. However, for broodstock shrimp during ovarian maturation and reproduction, as the active synthesis of ovarian nutrients, egg yolk compounds, sex steroids, and embryo development will consume considerable amounts of AA, much higher levels of dietary AA should be required. This has been confirmed in the current study, which showed that supplementation of at least 800 mg kg⁻¹ AAE to the basal diet was needed to bring excellent ovarian maturation and reproductive performance.

ACKNOWLEDGMENTS

The authors would like to acknowledge Mr. Chen Wenlin for kindly offering shrimp breeders and experimental facilities. We also thank Yuehai Feed Co. Ltd and Mr. Zheng Shixuan for offering feed ingredients and manufacturing facilities. Sincere thanks are also given to Mr. Cheng Kaimin and Mr. Zhou Qicun for their help in feed manufacturing. This work was funded by the Science Innovative Project (No KSCX2-1-04-04) from the Chinese Academy of Sciences.

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CHANGES IN THE BODY COMPOSITION OF ADULT MALE SOUTHERN ROCK LOBSTER, *JASUS EDWARDSII*, DURING STARVATION

L. E. MCLEOD, C. G. CARTER* AND D. J. JOHNSTON

School of Aquaculture, Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Locked Bag 1370, Launceston 7250, Tasmania, Australia

ABSTRACT This experiment determined the effect of starvation on the chemical composition and tissue histology of the abdominal muscle and digestive gland in adult male southern rock lobsters *Jasus edwardsii* during 14 and 28 days. Individual lobsters ($n = 6$) were stocked into 34-L tanks ($17.5 \pm 1.5^\circ\text{C}$) and either fed or starved over 14 and 28 days and also compared with lobsters ($n = 6$) killed for initial samples at the start of the experiment. Starved lobsters showed a significant reduction in crude lipid from the digestive gland at day 14 and from the abdominal muscle at day 28 when compared with the initial population. Histologic investigation of the digestive gland showed a lower density of lipid droplets in both day 14 and day 28 starved lobsters, with this depletion apparently causing structural damage to the digestive tubule in lobsters starved for 28 days. Histologic investigation of glycogen levels in digestive tubules showed a decline with starvation. Available energy was significantly affected by the crude lipid content of both organs although the digestive gland contained significantly higher absolute amounts. Starvation forces the use of body reserves to maintain metabolic functions; the order and quantity of depletion indicated the minimum requirements for survival and highlighted differences in the strategies used by crustaceans.

KEY WORDS: glycogen, histology, *Jasus edwardsii*, lipid, southern rock lobster, starvation

INTRODUCTION

The southern rock lobster *Jasus edwardsii* (Hutton, 1875) (Palaemonidae) is found along the coastlines of New Zealand and southern Australia and forms an important fishery in these regions (Thomas et al. 1998). Aquaculture is also under development in New Zealand and Australia; current strategies on-grow wild-caught puerulus, postpuerulus, and juveniles to market size (Jeffs & Hooker 2000, Thomas et al. 1998). Research into broodstock management and larval propagation is underway, and whereas there is a major focus on female broodstock and larval quality (Smith et al. 2003a, Smith et al. 2003b), little information is available on male rock lobsters.

Starved crustaceans, like all animals, must use body reserves to supply energy to maintain metabolism (Hervant et al. 1999, Lemmens 1994, Virtue et al. 1993), and the change in body composition indicates both the sequence and level of depletion of different body reserves. For decapod crustaceans there is conflict in the literature about the order and amount of reserve utilization and hence the role of protein, lipid, and carbohydrate during starvation. Many marine crustaceans metabolize lipid initially, using protein only after lipid reserves are depleted (Dawirs 1987, Regnault 1981). For example, rapid depletion of digestive gland lipid was documented in adult tiger prawns *Penaeus esculentus* (Barclay et al. 1983, Chandumpai et al. 1991), adult *Crangon crangon* (Regnault 1981), and in larval and adult shore crabs *Carcinus maenas* (Dawirs 1987, Heath & Barnes 1970). In contrast, larvae of the spider crab *Hyas araneus* (Anger & Dawirs 1982) and adult Antarctic krill *Euphausia superba* (Virtue et al. 1993) use body protein initially and lipids last. Therefore, assumptions about the use of body reserves as metabolic substrates by crustaceans during periods of nonfeeding or starvation should be avoided.

Lipid is used as the primary energy source to sustain onshore swimming activity of nonfeeding (starved) southern rock lobster puerulus prior to settlement (Jeffs et al. 1999, Jeffs et al. 2001, Lemmens 1994, Wells et al. 2001). Enzyme profiles also suggest

that lipid as well as carbohydrate are used by puerulus and that protein may be used for energy following depletion of these reserves (Johnston, 2003). Lipid is also a major source of energy in phyllosoma larvae with polar lipids and polyunsaturated fatty acids particularly important (Nelson et al. 2003). Energy utilization in broodstock is less understood, and only recently has the importance of broodstock diet been linked with phyllosoma quality and survival in hatcheries (Smith et al. 2003a). This study will be the first to examine utilization of energy reserves in male southern rock lobster broodstock through dedicated starvation experiments and will complete our understanding of energy utilization in the life-cycle of the southern rock lobster. The aims of this study were to measure and compare the standard growth parameters and chemical composition of the abdominal muscle and digestive gland in adult male southern rock lobsters (*J. edwardsii*) from an initial population and those that were fed or starved for 14 days and 28 days. Histology of the digestive gland has proved a sensitive indicator of nutritional condition (Johnston et al. 2003) and was used in the current study.

MATERIALS AND METHODS

Experimental Procedure

The experiment was conducted at the School of Aquaculture, University of Tasmania. Captive male southern rock lobsters (*J. edwardsii*) were obtained from Marine Research Laboratories (MRL), Hobart, Tasmania. This experiment was run during two time blocks with a total of 30 lobsters used. Each time block had an initial group, day 14 fed, day 14 starved, day 28 fed, and day 28 starved; each treatment consisted of 3 lobsters randomly allocated to individual experimental tanks. Prior to the commencement of each time block, lobsters were acclimatized to the system for 7 days. During acclimatization, any mortalities or molts were replaced with similar-sized animals from the stock population.

The system was maintained at $17.5 \pm 1.5^\circ\text{C}$ and kept dark to increase feeding time. Water exited the 12 experimental tanks via

*Corresponding author. E-mail: Chris.Carter@utas.edu.au

a standpipe and was returned to the biofilter with 3–5% replacement every 2 days. Water quality parameters (salinity, dissolved oxygen, ammonia, nitrate, and nitrite) were monitored to ensure water quality remained within limits recommended for spiny lobsters (Jeffs and Hooker 2000). At the start of each time block, an initial sample of three lobsters were placed on ice to induce a chill-coma and used for assessment of weight, carapace length, organ size, and chemical and histologic composition (see below). At the same time, weight and carapace length were measured in 12 lobsters that were then stocked individually into experimental tanks and randomly assigned into treatments (fed or starved over 14 or 28 days). Single animals were stocked to prevent effects of agonistic behavior and cannibalism (Thomas et al. 2003). Lobsters were fed mussel (*Mytilus edulis*) every 3 days at 1330, and uneaten food and waste was siphoned out the next morning. At the end of the experiment, lobsters were induced into a chill-coma and the carapace removed to reveal the digestive gland. Samples of digestive gland tissue were fixed immediately for histology. Digestive gland and abdominal muscle tissue was wrapped in foil and frozen at -40°C for freeze-drying and analysis of chemical composition (discussed in following section).

Chemical Analysis

Digestive glands and abdominal muscle were analyzed for chemical composition using standard methods to determine dry matter (freeze dry to constant weight); nitrogen (Kjeldahl using a selenium catalyst) (AOAC 1995); crude protein calculated using % nitrogen $\times 6.25$; crude lipid [Soxhlet apparatus and PET extraction (AOAC 1995)]; ash [muffle furnace at 550°C for 16 h (AOAC, 1995)]; and carbohydrate (nitrogen-free extract, NFE), determined by the difference between dry matter and sum of crude protein, lipid, and ash. Energy was calculated from the chemical composition using values of 23.6 kJ g^{-1} for protein, 38.6 kJ g^{-1} for lipid, and 17.2 kJ g^{-1} for carbohydrate (Jobling 1994).

Histology

Samples of digestive gland were dissected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature. Following washes in 0.1 M phosphate buffer (pH 7.4), samples were dehydrated in a graded series of ethanol and half the tissue post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h. Tissue was washed in 0.1 M phosphate buffer,

embedded in glycolmethacrylate (JB4) resin, sectioned at $2 \mu\text{m}$, and then stained with polychrome blue. The other half of each tissue sample was processed routinely for wax histology, sectioned at $5 \mu\text{m}$, then stained for glycogen using the periodic acid-Schiff (PAS) technique (Bancroft and Cook 1994). A negative diastase control was used to obtain more specificity in detecting glycogen (Humason 1972), and a positive control of trout mucus was initially stained with samples.

Statistical Analysis

Mean values are reported \pm standard error (SE). Each lobster sampled was assessed as a single replicate. The chemical composition values for digestive gland and abdominal muscle were the mean of two duplicates. Data were pooled to give six replicates for each of the five treatments (day 0, day 14 fed, day 14 starved, day 28 fed, and day 28 starved). Normality and homogeneity of variance were confirmed (SPSS version 8). Comparison between means was by one-way analysis of variance (ANOVA) and post-hoc comparison using Tukey's HSD (SPSS version 8). Significance was accepted at probabilities of 0.05 or less.

RESULTS

Chemical Composition

There were no significant differences in carapace length between the initial group and the different treatments nor were there were any significant differences in whole body wet weight, abdominal muscle index (ABI), or digestive gland index (DGI) between fed and starved lobsters (Table 1). However, starved lobsters showed a general trend of lower mean whole body weight and significantly different changes in wet body weight (Table 1). Starved lobsters tended to show a lower DGI, but multiple comparison was not sensitive enough to show which treatments were significantly different (Table 1).

Only crude lipid and gross energy content of abdominal muscle tissue were significantly different between treatments (Table 2). After 28 days of starvation, the abdominal muscle of lobsters had lower mean crude lipid than the abdominal muscle of lobsters in the initial population; no other significant changes were shown statistically. The correlations between chemical composition parameters of the abdominal muscle in fed and starved adult lobsters were investigated. Dry matter was positively correlated with crude

TABLE 1.
Growth and condition indices (% wet weight) of *J. edwardsii* (mean \pm SE, $n = 6$), when fed or starved for 14 or 28 days.

Day	Treatment					Statistical Analyses		
	0 Initial	14 Fed	14 Starved	28 Fed	28 Starved	df	f-value	P
Carapace length (mm)	118.63 ^a \pm 3.22	117.42 ^a \pm 1.00	118.42 ^a \pm 2.71	119.34 ^a \pm 2.48	118.74 ^a \pm 1.29	4, 25	0.631	0.645
Initial WBW (g)	804.62 ^a \pm 38.34	804.55 ^a \pm 41.25	804.65 ^a \pm 43.09	804.95 ^a \pm 24.80	804.58 ^a \pm 28.50	4, 25	0.746	0.570
Final WBW (g)	804.62 ^a \pm 38.34	860.17 ^a \pm 41.22	750.70 ^a \pm 24.26	786.05 ^a \pm 49.09	781.33 ^a \pm 25.07	4, 25	1.206	0.333
Change in WBW (g)	0 ^a	+55.62 ^a \pm 0.23	-53.95 ^b \pm 7.38	-18.90 ^c \pm 2.47	-23.25 ^c \pm 4.15	4, 25	105.6	0.0001
ABI (% BW)	14.45 ^a \pm 1.34	12.74 ^a \pm 0.58	13.30 ^a \pm 0.71	12.44 ^a \pm 0.59	12.52 ^a \pm 0.28	4, 25	1.195	0.338
DGI (% BW)	4.75 \pm 0.22	4.91 \pm 0.24	4.20 \pm 0.24	4.29 \pm 0.18	3.90 \pm 0.30	4, 25	2.859	0.044♣

Whole body weight WBW.

Abdominal muscle index ABI = (abdominal muscle wet weight g)/whole body wet weight g \times 100.

Digestive gland index DGI = (digestive gland wet weight g)/whole body wet weight g \times 100.

Values with a different superscript were significantly different ($P < 0.05$).

♣ Multiple comparisons were not sensitive enough to identify significantly difference mean values.

TABLE 2.

Tissue composition (% wet weight) of *J. edwardsii* (mean \pm SE, $n = 6$), abdominal muscle and digestive gland fed or starved for 14 or 28 days.

Day	Treatment					Statistical Analyses		
	0 Initial	14 Fed	14 Starved	28 Fed	28 Starved	Df	F-value	P
Abdominal muscle								
Dry matter	26.32 ^a \pm 0.47	26.88 ^a \pm 0.37	25.33 ^a \pm 0.46	25.59 ^a \pm 0.33	25.67 ^a \pm 0.47	4, 25	1.006	0.423
Crude lipid	2.53 ^a \pm 0.14	2.38 ^{ab} \pm 0.67	1.96 ^{ab} \pm 0.14	1.84 ^{ab} \pm 0.05	1.20 ^b \pm 0.28	4, 25	3.20	0.030
Crude protein	23.27 ^a \pm 0.42	23.63 ^a \pm 0.36	22.07 ^a \pm 0.27	22.32 ^a \pm 0.30	22.30 ^a \pm 0.70	4, 25	2.28	0.89
Carbohydrate ¹	0.06 ^a \pm 0.06	0.06 ^a \pm 0.06	0.25 ^a \pm 0.09	0.40 ^a \pm 0.10	1.13 ^a \pm 0.64	4, 25	1.555	0.217
Ash	1.73 ^a \pm 0.05	1.45 ^a \pm 0.10	1.65 ^a \pm 0.03	1.43 ^a \pm 0.12	1.52 ^a \pm 0.10	4, 25	1.690	0.184
Gross energy ² (kJ.g ⁻¹)	6.24 ^a \pm 0.19	6.42 ^a \pm 0.23	5.94 ^a \pm 0.27	5.91 ^a \pm 0.18	5.87 ^a \pm 0.22	4, 25	2.879	0.043♣
Digestive gland								
Dry matter	42.71 ^a \pm 0.94	44.55 ^a \pm 1.28	43.98 ^a \pm 2.15	43.57 ^a \pm 1.63	41.99 ^a \pm 2.63	4, 25	0.590	0.674
Crude lipid	30.04 ^a \pm 0.53	30.68 ^{ab} \pm 1.69	20.58 ^b \pm 2.20	28.80 ^{ab} \pm 1.86	22.72 ^{ab} \pm 1.68	4, 25	7.798	0.0001
Crude protein	11.16 ^a \pm 0.40	11.77 ^a \pm 0.38	12.09 ^a \pm 0.02	11.11 ^a \pm 0.37	11.34 ^a \pm 0.22	4, 25	1.817	0.157
Carbohydrate ¹	0.86 ^a \pm 0.40	0.96 ^a \pm 0.40	9.71 ^b \pm 2.72	2.32 ^{ab} \pm 0.73	7.30 ^{ab} \pm 3.17	4, 25	4.449	0.007
Ash	1.45 ^a \pm 0.02	1.46 ^a \pm 0.03	1.55 ^a \pm 0.10	1.70 ^a \pm 0.08	1.75 ^a \pm 0.07	4, 25	0.051	0.995
Gross energy ² (kJ.g ⁻¹)	12.96 ^a \pm 0.33	14.68 ^a \pm 0.67	13.77 ^a \pm 0.43	14.34 ^a \pm 1.09	12.72 ^a \pm 0.49	4, 25	1.979	0.129

¹ Carbohydrate was calculated by difference (Dry matter – (crude protein – crude lipid – ash)).

² Energy was calculated using values of 23.6 kJ.g⁻¹ for protein, 38.6 kJ.g⁻¹ for lipid and 17.2 kJ.g⁻¹ for carbohydrate (Jobling, 1994).

Values which share the same superscript are not significantly different.

♣ Multiple comparisons were not sensitive enough to identify significantly difference mean values.

protein ($r = 0.50$, $P \leq 0.01$), and energy content ($r = 0.69$, $P \leq 0.01$), and energy content was positively correlated with crude protein ($r = 0.55$, $P \leq 0.01$) and crude lipid ($r = 0.37$, $P \leq 0.05$).

Crude lipid content of the digestive gland was significantly lower in day 14 starved lobsters than at day 0 (Table 2). There were no other significant differences between treatments although crude lipid in day 28 lobsters was suggestive of the same trend. Carbohydrate was significantly higher in day 14 starved lobsters compared with day 14 fed and the day 0 group. There were no significant differences in dry matter, crude protein, ash, or energy. Dry matter was not correlated with crude lipid ($r = 0.15$, $P > 0.05$) or carbohydrate ($r = 0.16$, $P > 0.05$), and crude lipid was not correlated with digestive gland energy content ($r = 0.15$, $P > 0.05$). There was a significant negative correlation between crude lipid and carbohydrate ($r = -0.43$, $P \leq 0.05$).

Histologic Examination of the Digestive Gland

Lipid droplets in epithelial cells of the digestive gland tubules from the initial lobster population were densely arranged in the apical cytoplasm and less dense adjacent to the basal lamina (Fig. 1A). The epithelia of tubules from lobsters fed for 14 days had densely arranged lipid droplets that were spaced throughout the cytoplasm (Fig. 1B). Epithelial cells in lobsters starved for 14 days had a large number of empty vacuoles concentrated apically with densely arranged lipid droplets in the cytoplasm adjacent to the basal lamina (Fig. 1C). This agrees with the reduction in crude lipid found in the chemical composition data. Lobsters fed for 28 days had tubules with densely arranged lipid droplets, similar to the 14-day fed lobsters. Epithelial cells of lobsters starved for 28 days had large amounts of empty vacuoles apically around the tubule lumen, with both loosely arranged lipid droplets and empty vacuoles adjacent to the basal lamina and outer membranes ruptured (Fig. 1E).

The digestive gland epithelial cells in the initial lobster population had minimal glycogen granular material (Fig. 2A). The

negative control, diastase treatment was used over all treatments and all had less intense staining, indicating stained material was glycogen (Figs. 2B, 3D, 4B, and 4D). Epithelial cells from lobsters fed for 14 days had intense positive staining for glycogen (Fig. 3A) whereas starved lobsters had fewer positive staining granular bodies (Fig. 3C). Cells from lobsters fed for 28 days had less glycogen (Fig. 4A) than 14-day fed lobsters, although cells from lobsters starved 28 days had a much lower quantity of glycogen than 14 day starved lobsters (Fig. 4C).

DISCUSSION

Chemical Composition

The nonsignificant trend of lower whole body weight in starved lobsters is consistent with body tissue being used during food deprivation to maintain metabolism (Hervant et al. 1999, Lemmens 1994, Virtue et al. 1993). However, unlike vertebrates, crustacean body volume is set by their rigid exoskeleton (Stuck et al. 1996), therefore to maintain this set volume any change in body mass is counteracted by a change in water content (Barclay et al. 1983, Hervant et al. 1999, Stuck et al. 1996). The mass of digestive gland and abdominal muscle as a percentage of whole body weight showed opposite changes with starvation, which support conclusions that organs respond differently to nutritional stress (Schirf et al. 1987).

The total wet weight and percent dry matter of abdominal muscle showed no obvious or significant trends when lobsters were starved. However, the reduction in percent dry matter of abdominal muscle in lobsters fed for 28 days indicates possible catabolism of muscle tissue. This, in addition to the increases in uneaten feed recovered from tanks of day 28 fed lobsters, indicated they had entered premolt shortly after day 14 and were close to ecdysis at time of sampling (day 28). Crustaceans stop feeding during early premolt and use body reserves until the shell hardens

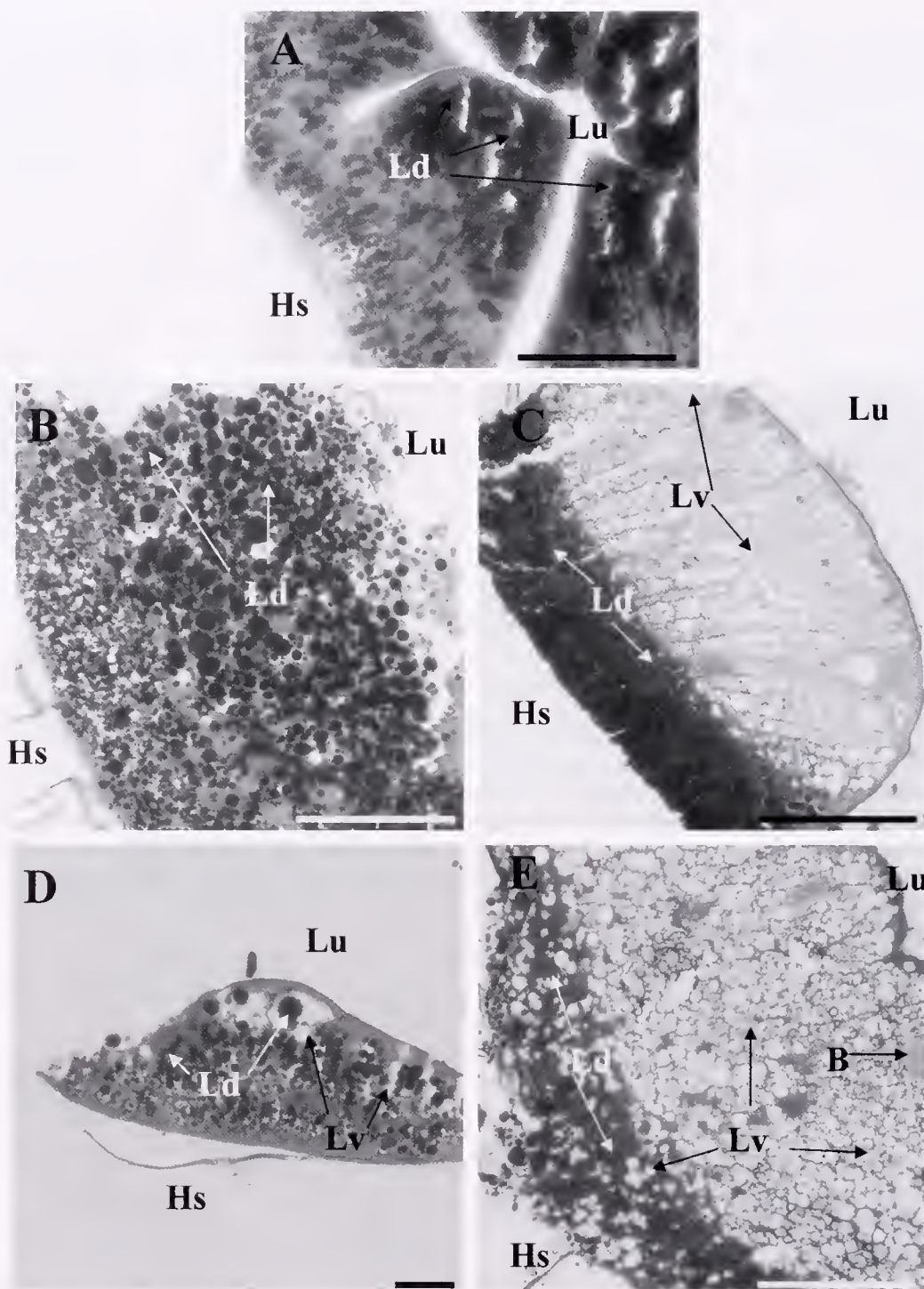


Figure 1. Transverse sections (TS) of a digestive gland tubule from *J. edwardsii*. Tissue post-fixed in osmium for detection of lipid. (A) Initial population. (B) Day 14 fed lobster. (C) Day 14 starved lobster. (D) Day 28 fed lobster. (E) Day 28 starved lobster. Scale bar, 50 μ m. (Hs, hemolymph space; Lu, tubule lumen; Ld, lipid droplets; Lv, empty lipid vacuole; B, B-cell vacuole.)

(Barclay et al. 1983, Chang & O'Connor 1983, Factor 1995, Harrison 1997, Hiller-Adams & Childress 1983, Jones et al. 1997).

Although abdominal muscle crude protein showed a small range (22.0–23.7% wet weight) between treatments, it was positively correlated with the percent dry matter ($P \leq 0.01$); a similar correlation was also found in Pacific white shrimp *Penaeus vannamei* (Stuck et al. 1996). Starved lobsters tended to have a lower

abdominal muscle percent crude protein than lobsters fed over the same time period, a trend also found in adult tiger prawns when starved for 14 days (Barclay et al. 1983) and in small adult *J. edwardsii* lobsters (80–90 mm carapace length) fed once every 10 days (Musgrove 1997). Lobsters fed for 28 days showed lower percent crude protein than lobsters from day 0 and day 14 fed, and this reduction is explained by muscle protein decreasing to permit

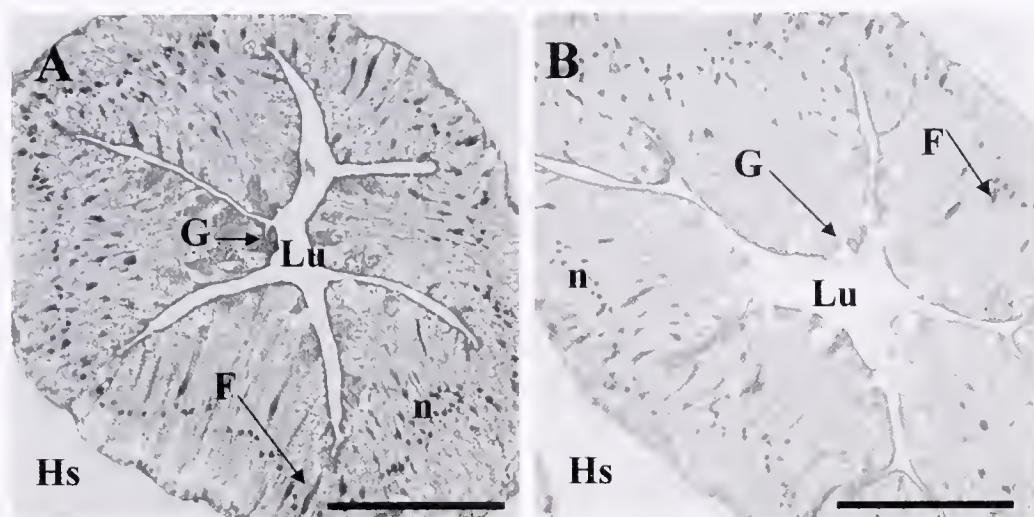


Figure 2. TS of digestive gland tubules of *J. edwardsii*, stained with PAS for glycogen. (A) Initial population. (B) Diastase control for initial population. Scale, 100 μ m. (Hs, hemolymph space; Lu, tubule lumen; n, nucleus; F, F-cell; B, B-cell; G, glycogen.)

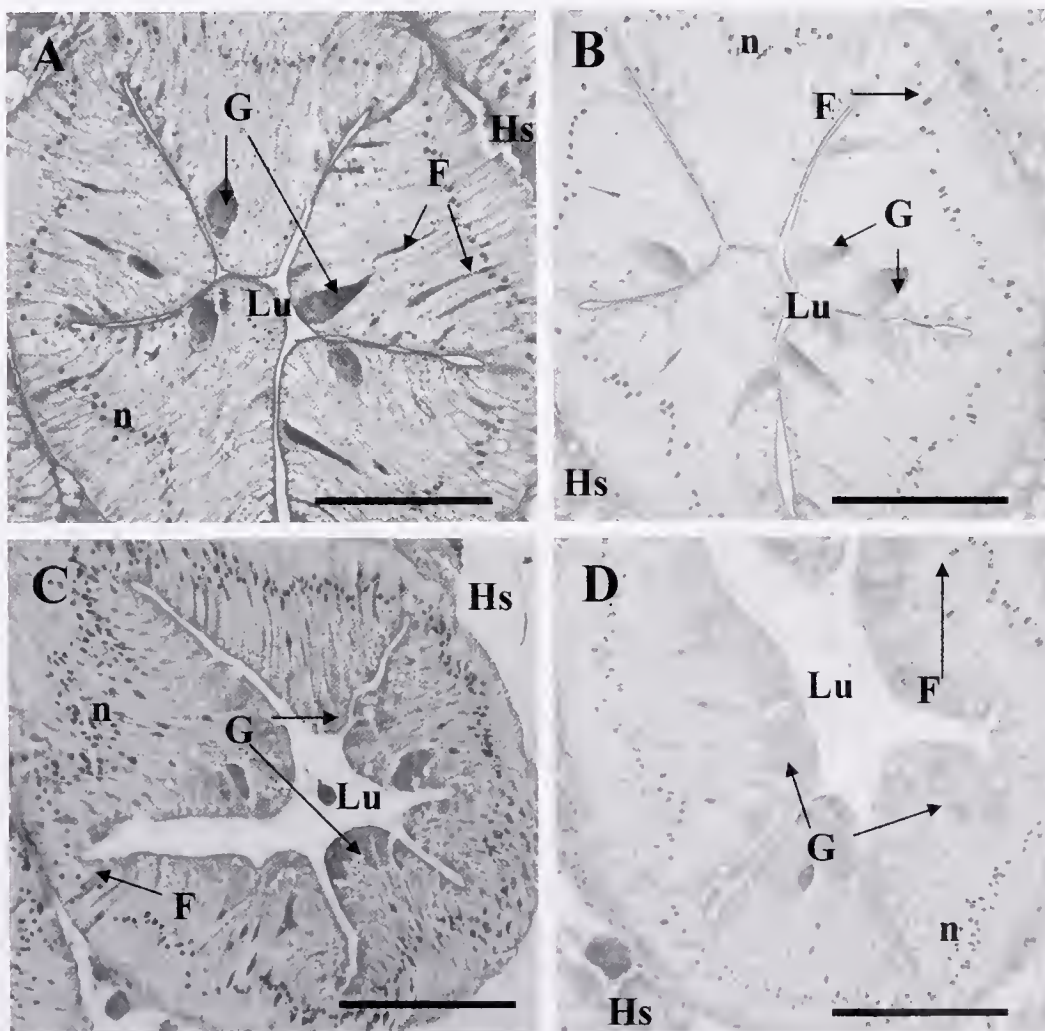


Figure 3. TS of digestive gland tubules in *J. edwardsii*, stained with PAS for glycogen. (A) Day 14 fed lobster. (B) Diastase control for day 14 fed lobster. (C) Day 14 starved lobster. (D) Diastase control for day 14 starved lobster. Scale, 100 μ m. (Hs, hemolymph space; Lu, tubule lumen; n, nucleus; F, F-cell; G, glycogen.)

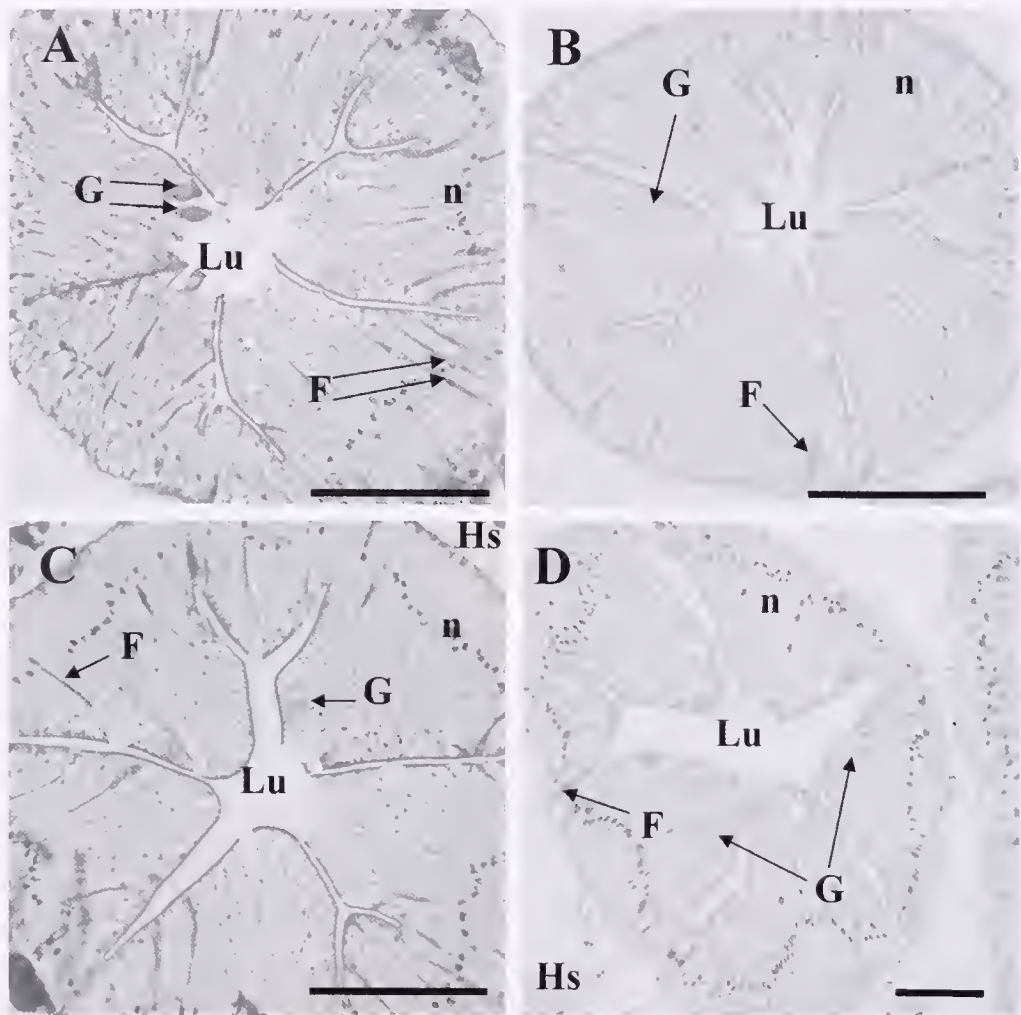


Figure 4. TS of digestive gland tubules of *J. edwardsii*, stained for glycogen. (A) Day 28 fed lobster. (B) Diastase control for day 28 fed lobster. (C) Day 28 starved lobster. (D) Diastase control for day 28 starved lobster. Scale, 100 μ m. (Hs, hemolymph space; Lu, tubule lumen; n, nucleus; F, F-cell; G, glycogen.)

the escape of lobster from the old exoskeleton during the molt cycle (Chang & O'Connor, 1983). The significantly lower lipid level in the abdominal muscle of starved lobsters at 28 days compared with the initial population is consistent with findings in starved adult tiger prawns (Chandumpai et al. 1991). The significant correlation between percent crude lipid and abdominal muscle energy content indicates that although the mean absolute wet weight of crude lipid stored in the abdominal muscle was less than 0.4% whole body wet weight, changes still affected the energy matrix within the abdominal muscle during starvation, which somewhat conflicts with suggestions that the low levels of lipid in the lobster muscle are mostly structural and are not normally available for energy production (Dall 1981).

The trend of lower percent dry matter in the digestive gland of starved lobsters, indicating a reduction of total body stores, further supports the digestive gland being the primary organ for energy storage in decapod crustaceans (Dall 1981, Gibson & Barker 1979, Icely & Nott 1992). The percent crude protein of digestive gland increased in starved lobsters as has been found in the digestive gland of the marine carnivorous prawn *Pandalus platycero* (Whyte et al. 1986). However, the apparent increase in crude protein content is due to the reduction in digestive gland tissue mass rather

than an increase in the amount of protein. In general, decapod crustaceans have higher digestive gland lipid than most other groups (Gibson & Barker 1979). This study found large adults (117.42–119.34 mm carapace length) had a crude lipid content of 47–71% digestive gland dry weight and within the range for juvenile southern rock lobsters in some studies (Crear et al., 2001) but not others (Johnston et al. 2003, Ward et al. 2003). Generally, the digestive gland lipid content in the current study was considerably higher than in other decapod crustaceans (Barclay et al. 1983, Chandumpai et al. 1991). Digestive gland crude lipid also appears to vary within the lobster family: 40–50% for adult *J. lalandii* (carapace lengths 70–89 mm) (Cockcroft 1997); 17–65% for adult *Homarus americanus* during 102 days of starvation (Stewart et al. 1972); and <12% for adult *J. edwardsii* (Musgrove 1997). The absolute amount of crude lipid was higher in the digestive gland (6.41–12.96 g) than in the abdominal muscle (1.17–2.94 g), which further emphasizes the importance of the digestive gland as an energy store in crustaceans (Cockcroft 1997, Dall 1981, Jeffs et al. 2001).

The increase in the estimated carbohydrate levels of digestive gland in lobsters fed for 28 days may be explained by the increase in total glycogen deposited in digestive gland and epidermis during

the transition of spiny lobsters into premolt (Schwabe et al. 1952). Although later publications have challenged some of the conclusions from this paper (Dall 1981, Stewart et al. 1972), the increase in digestive gland glycogen was also found to be significantly higher in prawn *P. kerathurus* before molting (Hilmy et al. 1986). The negative correlation between carbohydrate and crude lipid in the digestive gland may be explained by the suggestion that carbohydrates act as an intermediate energy substrate following the breakdown of other storage products such as lipids (Jeffs et al. 1999) and may also explain increased content due to the breakdown of lipid. The method of carbohydrate quantification used was to calculate the difference between dry matter and the other measured tissue macro-chemical components and gave estimated ranges of 1.6–42.7 and 56.4–212.0 mg/g dry tissue for the abdominal muscle and digestive gland tissues, respectively. The carbohydrate content of the digestive glands from the day 14 and day 28 starved lobsters were 212 and 140 mg/g dry tissue, respectively, and appeared very high in comparison to other samples in the current study and to some other studies on crustaceans (Hilmy et al. 1986, Whyte et al. 1986). Digestive gland carbohydrate varied between 17 and 47 mg/g dry material, depending on dietary carbohydrate, in small (<10 g) *J. edwardsii* (Johnston et al. 2003). However, higher values have been reported in the digestive gland of lobsters including 168 mg/g dry tissue for adult (470 g) *Homarus americanus* (Floreto et al. 2000). The results from the current study indicate that further investigation of carbohydrate dynamics using direct measurements would be informative and also that the histologic approach used in the current study went some way to addressing this issue. The histologic assessment of glycogen is clearly more sensitive than estimating carbohydrate by difference (see below).

Histology

The prevalence of empty lipid vacuoles in digestive gland epithelial cells of unfed lobsters (Figs. 1C and E) confirms that lipid is used by *J. edwardsii* broodstock during periods of starvation and supports the reduction of lipid content in the digestive gland of starved lobsters as determined by chemical analysis. Tissue histology also shows that short-term depletion of lipid reserves has no effect on structure of the digestive gland tubules, which remain unchanged in lobsters starved for 14 days. But after 28 days of starvation, when lipid reserves are near exhausted, outer cell mem-

branes are degraded and the cytoplasm is diffuse. Such tissue damage was also observed during periods of nutritional deprivation in adult freshwater crayfish marron *Cherax tenuimanus* (Jussila 1997). Although change in the number and size of B-cells or R-cells is used as an indication of nutritional stress (Al-Mohanna and Nott 1987, Niles et al. 1993), no such differences were apparent between the treatments in this trial.

Glycogen was also depleted from digestive gland epithelia during starvation with greater depletion from tissues the longer lobsters were starved (Figs. 3C and 4C). Although it is widely accepted that digestive gland glycogen reserves are rapidly depleted over a period of nonfeeding in crustaceans (Dall 1986, Gibson & Barker 1979, Hilmy et al. 1986, Jeffs et al. 2001), this is one of the few studies to demonstrate this fact using tissue histology. The higher intensity staining in the day 14 fed lobsters indicates they are in better nutritional condition than lobsters from the initial population and agrees with chemical composition data. It is unclear why lobsters fed for 28 days had lower glycogen than lobsters fed for 14 days, as 28 days fed animals had higher levels of carbohydrate, as determined by difference. It is possible this discrepancy may be due to resorption of glycogen by day 28 lobsters for chitin synthesis as they were entering premolt during the latter stages of the experiment (Aiken 1980, Chang, 1995). This physiologic mechanism may not be apparent from the chemical composition data, which only estimated total carbohydrate.

CONCLUSIONS

This study has confirmed the importance of lipid and glycogen as energy sources for southern rock lobster broodstock using the combination of chemical analyses and tissue histology. Research on starvation effects in southern rock lobster over longer time periods may show more of the significant differences between fed and starved animals found in other crustaceans. There appears to be a relationship between crustacean body mass and the energy reserve composition, this may indicate a switch in dietary needs with carapace length. The increase in the weight of apparent carbohydrate in both the abdominal muscle and digestive gland during starvation is cause for investigation as these results conflict with the reduction of body reserves during starvation. The results from this study, together with further research into the effect of starvation on southern rock lobster biochemical composition, should aid in the development of understanding of the importance of individual energy reserves.

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THE LIPID COMPOSITION OF EARLY STAGE WESTERN ROCK LOBSTER (*PANULIRUS CYGNUS*) PHYLLOSOMA: IMPORTANCE OF POLAR LIPID AND ESSENTIAL FATTY ACIDS

GRANT C. LIDDY,¹ MATTHEW M. NELSON,² PETER D. NICHOLS,³ BRUCE F. PHILLIPS¹
AND GREG B. MAGUIRE⁴

¹Aquatic Science Research Unit, Curtin University of Technology, Perth, W.A. 6845, Australia;

²Department of Zoology, University of Tasmania, Hobart, Tasmania, 7001, Australia; ³CSIRO Marine Research, Hobart, Tasmania, 7001, Australia; ⁴Department of Fisheries, Research Division, North Beach, W.A. 6920, Australia

ABSTRACT Total lipid, lipid class, and fatty acid analyses were conducted on fed and starved stage I and II phyllosoma of the western rock lobster *Panulirus cygnus*. In both stages, the decrease in dry mass of starved larvae and increase in dry mass of *Artemia*-fed larvae were accompanied by a decrease and increase in lipid content, respectively. Lipid accounted for 6.7% of the decrease in dry mass in starved stage I larvae, which increased to 35.0% in stage II larvae. Also, lipid accounted for 6.2% of the increase in dry mass of fed stage I larvae, increasing to 19.2% in stage II larvae. The major lipid classes in all phyllosoma samples were polar lipids (84.1–94.3%) followed by sterols (6.6–12.1%; mainly cholesterol). Gravimetrically, fed larvae increased predominantly in polar lipid whereas in starved larvae, polar lipid was the major lipid class catabolized, with the sterol content not changing significantly. Hydrocarbons, wax esters, diacylglycerol ether, triacylglycerols, and free fatty acids were all minor lipid classes (<5% of total lipid). Fatty acid analysis showed six major components present: 16:0, 18:1n-9, 18:0, 20:4n-6 (arachidonic acid; AA), 20:5n-3 (eicosapentaenoic acid; EPA) and 22:6n-3 (docosahexaenoic acid; DHA). These fatty acids all increased gravimetrically in fed larvae and decreased in starved larvae. In starved larvae, small decreases were seen in the relative contribution of EPA, DHA, 16:1n-7, and 18:1n-9, with AA increasing. In fed larvae, most of the major fatty acids remained at a similar relative level, and larvae were able to accumulate AA and EPA, but not DHA, above the relative level (%) in *Artemia*. The results are useful in the identification of nutrients required during development and as such with the design of diets used in phyllosoma culture.

KEY WORDS: *Artemia*, fatty acids, lipid, *Panulirus cygnus*, phyllosoma, polar lipid, rock lobster

INTRODUCTION

With increased product demand and value, aquaculture or enhancement of spiny lobster fisheries is currently receiving considerable interest due to wild fisheries being fully exploited worldwide (Kittaka 1994, Phillips et al. 2000). The western rock lobster, *Panulirus cygnus*, is found on the lower west coast of Australia and supports the world's largest rock lobster fishery (Phillips et al. 2000). To ensure sustainability, a major spiny lobster aquaculture industry would need to be based on culture of phyllosoma from hatching through their entire larval development (Crear et al. 1998, Kittaka and Booth 1994). A major hurdle in complete culture of spiny lobsters is the long and complex larval component of its life history. Although the larval life cycle has been completed in the laboratory for a number of species, only limited success has been achieved (e.g., Booth 1995, Kittaka 1997), with many studies experiencing high mortality in early stages (e.g., Kittaka et al. 1997, Kittaka 1988, Kittaka & Ikegami 1988).

Nutrition is regarded as a key factor controlling survival and growth in crustacean larval culture (Mikami et al. 1995). Very little is known about the feeding of phyllosoma in the wild, creating a need for larval nutritional research, as nutritional studies on spiny lobsters (Palinuridae), particularly on the larval stages that are difficult to rear, are scarce (Kanazawa and Koshio 1994, Phleger et al. 2001). Phyllosoma have been reared on *Artemia* and mussel gonad with varying success (Illingworth et al. 1997, Kittaka 1988), however, appropriate nutrition requires the identification of the essential elements within the diet (Kanazawa and Koshio 1994). Lipid has been found to be of prime importance in crustacean larval stages (Kattner et al. 1994, Sasaki et al. 1986), and appears to be the main storage product in late stage phyllosoma, which is then used as an energy source during the nonfeeding puerulus stage (Jeffs et al. 1999, Jeffs et al. 2001).

Starvation experiments are one means to determine nutritional requirements of fish and crustacean larvae, with the retention of specific fatty acids (FA) during starvation being interpreted as a requirement for that specific FA (Olsen 1998). Koven et al. (1989) found fish larvae (*Sparus aurata*) conserved essential *n*-3 FA during starvation, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and considered this to be a reasonable biochemical strategy as these FA are more valuable as components of membranes than as energy sources. Biochemical studies during starvation and feeding of prawn larvae have also supplied valuable information on the best nutrient specification of food for the larvae (D'Souza 1998).

The objective of this work was to examine which lipids (lipid classes and fatty acids) were of greater nutritional importance for stage I and II *P. cygnus* phyllosoma by comparing patterns of conservation and loss in starved and fed larvae. The aim was to provide information that would be useful in defining suitable diets with profiles appropriate for phyllosoma culture.

MATERIALS AND METHODS

Broodstock

The study was undertaken at the Western Australian Marine Research Laboratories, Perth, Australia. Broodstock animals were kept in the laboratory under 12L:12D light cycle and fed daily with live mussels (*Mytilus edulis*) and routinely with fish and abalone. Larvae used in the stage I experiment hatched from a lobster (128.3 mm carapace length) mated in the laboratory, with oviposition of eggs (24 May 2001) at 25°C and incubation in a tank at 22°C (32–34 ppt), with larvae hatching after 39 days incubation. Larvae used in the stage II experiment hatched from a lobster (105.0 mm carapace length) mated in the laboratory, with ovipo-

sition of eggs (29 July 2001) at 25°C and incubation in a tank at 19°C (32–34 ppt), with larvae hatching after 57 days incubation.

Experimental System

The system used for rearing the phyllosoma has successfully been used to rear southern rock lobster (*Jasus edwardsii*) phyllosoma to their final stage (Ritar 2001). Seawater used in the experiments was heated to 23°C, filtered to 1 µm and UV sterilized (UViVF-9, 30 W) before entering the circular 30-L plastic rearing tubs. Water entered the tubs through four equally spaced nozzles (jets) positioned close to the bottom perimeter of the tub, with another two toward the bottom center of the tub to provide a circular water flow, thereby keeping phyllosoma moving in the water column. Each tub had a water flow of approximately 1 L/min, and the volume was maintained at 10 L. Excess water exited through screens positioned on the side of the tubs. Phyllosoma were fed daily with *Artemia* at 3/mL. Every morning, remaining *Artemia* were removed from the system by replacing the usual screens, "feeding filters" (200 µm), with "cleaning filters" (1500 µm) for approximately 1 h that allowed the *Artemia* to be flushed out of the tubs. The feeding filters were replaced and freshly enriched *Artemia* were then added to the tubs. Phyllosoma were transferred to clean tubs weekly. Phyllosoma that were starved during stages I and II received the same daily procedure except they were not fed. Larvae starved in stage II had been fed in stage I. The number of larvae added to the tubs was estimated volumetrically, and larvae were randomly stocked at approximately 1,500/tub.

Artemia

Phyllosoma were fed with *Artemia* (Great Salt Lake) that had been on-grown for 4–5 days, after reaching instar II, using Algamac 2000 (Biomarine, Aquafaruna) and an *Isochrysis* marine algal concentrate (Reed Mariculture, San Jose, CA). *Artemia* were also enriched for 18 h (two feedings, 1600 and 0200) with Algamac 2000 prior to feeding to the phyllosoma.

Sampling Protocol

All phyllosoma samples for biochemical analysis were taken in triplicate (i.e., three tubs were used for each sample). For stage I analyses, phyllosoma were sampled at hatch (day 0), fed and starved samples in the middle of stage I (day 6), and a sample of fed larvae after molting at the beginning of stage II (day 15). For stage II analysis, phyllosoma were sampled at hatch (day 0), at the beginning of stage II (day 15), fed and starved samples were taken in the middle of stage II (day 20), and a sample of fed larvae after molting at the beginning of stage III (day 26). Larval stages were measured from the anterior margin of the cephalic shield between the eyestalks to the posterior of the abdomen and staged according to Braine et al. (1979). Samples of enriched *Artemia* were taken for analysis.

Lipid Analysis

Lipid Extraction

Phyllosoma and *Artemia* samples were filtered onto 47-mm Whatman GFC filters and washed with 0.5 M ammonium formate. Samples were stored at –80°C, freeze-dried overnight, and weighed to determine dry mass (DM). Samples were quantitatively extracted overnight using a modified Bligh and Dyer (1959) one-

phase methanol:chloroform:water extraction (2:1:0.8 v/v/v). The phases were separated the following day by the addition of chloroform and water to give a final solvent ratio of 1:1:0.9 v/v/v methanol:chloroform:water. The total solvent extract (TSE) was concentrated using a rotary evaporator at 40°C, blown down to dryness under nitrogen, and weighed to determine the total lipid content. Samples were made up in a known volume of chloroform and stored at –20°C before analysis.

Lipid Classes

To quantify individual lipid classes (LC), an aliquot of the TSE was analyzed using an Iatroscan MK V TH 10 thin-layer chromatography-flame ionization detector (TLC-FID) (Iatron Laboratories, Tokyo, Japan). Samples were applied in duplicate to silica gel SHH chromarods (5-µm particle size) using 1-µL disposable micropipettes. Chromarods were developed in a glass tank lined with pre-extracted filter paper. The primary solvent system used for lipid class separation was hexane:diethyl ether:acetic acid (60:17:0.1 v/v/v), a mobile phase resolving nonpolar compounds such as wax esters (WE), triacylglycerols (TAG), free fatty acids (FFA), and sterols (ST). A second nonpolar solvent system of hexane:diethyl ether (96:4 v/v) was also used to resolve hydrocarbons (HC) from WE, and TAG from diacylglycerol ether (DAGE). After development, the chromarods were oven-dried and analyzed immediately. The FID was calibrated for each compound class (phosphatidylcholine, cholesterol, cholesteryl ester, oleic acid, squalene, TAG [derived from fish oil], WE [derived from fish oil] and DAGE [derived from shark oil], 0.1–10 µg range), and the peaks were quantified using DAPA software (Kalamunda, Western Australia).

Fatty Acids

An aliquot of the TSE was *trans*-methylated to produce fatty acid methyl esters (FAME) using methanol:chloroform:conc. hydrochloric acid (10:1:1 v/v/v) at 80°C for 2 h. The FAME produced were extracted into hexane:chloroform (4:1 v/v, 3 × 1.5 mL), reduced under nitrogen to dryness, and treated with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, 100 µL, 70°C, overnight) to convert ST and alcohols to their corresponding TMSi (trimethylsilyl) ethers. Samples were blown down under nitrogen and an internal standard (C19 and C23, 40 mg/g) was added.

Gas chromatographic (GC) analyses were performed with a Hewlett Packard 5890A GC (Avondale, PA) equipped with a HP-5 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.), a FID, a split/splitless injector, and a HP 7673A auto sampler. Helium was used as the carrier gas. Samples were injected in splitless mode at an oven temperature of 50°C. After 1 min, the oven temperature was raised to 150°C at 30°C/min, then to 250°C at 2°C/min, and finally to 300°C at 5°C/min. Peaks were quantified with Waters Millennium software (Milford, MA). Individual components were identified using mass spectral data and by comparing the retention time data with those obtained for authentic and laboratory standards. GC results are subject to an error of ±5% of individual component area. GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-mass spectrometer (Austin, TX) fitted with an on-column injector. The GC was fitted with a capillary column similar to that described above.

Statistical Analysis

Results were analyzed using one-way ANOVA with Tukey's test used for multiple comparisons. Percentage data was arcsine√

transformed and gravimetric data $\sqrt{}$ transformed to make the data normal and homogenous. When two samples were compared (hatch sizes), a *t*-test was used. Statistical analyses were performed using Statistica software (StatSoft Inc., Tulsa, OK, version 6). Data is presented as mean \pm SD, and results were considered significantly different at $P \leq 0.05$.

RESULTS

Larvae from the two batches did not significantly differ in DM or lipid content (mg/g DM and μ g/phyllsoma) at hatch; however, the lipid content was slightly elevated in larvae from the second hatch. For stage I samples, changes in fed and starved larvae were compared with newly hatched larvae (from first hatch), and for stage II samples to newly molted stage II larvae (from the second hatch).

Phyllsoma and Lipid Amounts

By mid stage, the DM had increased in stage I and II (significant) fed larvae (16.5 and 33.6%, respectively) and conversely decreased in starved stage I (significant) and II larvae (29.1 and 10.9%, respectively) (Table 1). Larvae also showed increases in dry mass (DM) by the time they molted to the next stage (Table 1). As with DM, the lipid content (mg/g DM and μ g/phyllsoma) of stage I and II larvae increased in fed samples and decreased in starved samples (Table 1). In stage I larvae, the increase in lipid content of fed larvae accounted for 6.2% of the increase in DM whereas the lipid decrease in starved larvae accounted for 6.7% of the decrease in DM. In stage II larvae, lipid accounted for a much greater percentage of the increase in DM of fed larvae, 19.2%, and also accounted for a much larger portion of the decrease in starved stage II larvae, 35.0%. Although newly molted stage III did have a similar amount of lipid/phyllsoma compared with fed stage II larvae, total lipid (mg/g DM) decreased significantly after the molt (Table 1).

Lipid Class

In all phyllsoma samples, polar lipid (PL) was the major LC (82.0–94.3%) (Table 2). As with lipid amounts, PL concentration in larvae differed from the different hatches, larvae from the second hatch (49.1 mg/g DM) having significantly increased levels compared with larvae from the first hatch (37.6 mg/g DM) (Table

2, Fig. 1). PL was also the major LC used during starvation and on an absolute basis was significantly reduced in starved stage I and II larvae (Table 2, Fig. 1). The amount of PL in starved stage I larvae (27.2 mg/g DM) was 27.6% less than in newly hatched larvae (37.6 mg/g DM), and increased by 8.4% in fed phyllsoma (40.8 mg/g DM) (Fig. 1). For stage II larvae, the amount of PL in starved larvae (29.1 mg/g DM) was 53.9% less than in newly molted stage II larvae (63.0 mg/g DM), and increased by 50.2% in fed stage II larvae (94.7 mg/g DM) (Fig. 1). In both stages, the increase in PL (mg/g DM) almost solely accounted for the increase in lipid (mg/g DM). ST were the next most abundant lipid class (largely cholesterol) comprising between 5.1–12.1% of the total lipid (Table 2). In relative proportions, ST levels (%) increased in starved larvae (significant in stage II), with no significant decrease in amount (mg/g DM) (Table 2, Fig. 1). HC were the next most abundant LC (0.4–4.6%), followed by WE (0.1–2.3%), FFA (0.1–1.1%), DAGE and TAG ($\leq 0.2\%$) (Table 2).

Enriched *Artemia* contained high relative and absolute levels of PL (62.0%; 83.9 mg/g DM), although unlike the phyllsoma samples, also had high levels of TAG (31.0%; 41.8 mg/g DM). This was followed by ST (3.3%; 4.5 mg/g DM), and FFA (3.1%; 4.2 mg/g DM), with HC, DAGE, and WE being minor components ($\leq 0.3\%$; < 0.5 mg/g DM) (Table 2, Fig. 1).

Fatty Acids

Gravimetrically, newly hatched larvae from the second hatch were significantly elevated in total FA and all the major FA compared with the first hatch (Tables 3 and 4, Fig. 2). Of the 55 fatty acids (FA) identified, 18 individual FA had some concentrations $\geq 1\%$ in the phyllsoma samples. The six most abundant FA ($\geq 5\%$) were 20:5n-3 (EPA, 13.7–21.3%), 16:0 (palmitic acid, 10.7–15.2%), 18:1n-9c (oleic acid, 10.6–14.6%), 22:6n-3 (DHA, 7.1–13.0%), 20:4n-6 (arachidonic acid, AA, 5.8–12.4%), and 18:0 (stearic acid, 8.5–13.9%) (Tables 3 and 4). These six components generally accounted for between 68.6 to 76.9% of the total FA.

The major FA in fed stage I larvae showed similar relative contributions to newly hatched larvae, except there was a significant increase in EPA and decrease in 16:0 (Table 3). Similarly, fed stage II larvae showed a similar relative contribution of the major FA compared with newly molted stage II larvae, although 16:0 was significantly elevated (Table 4). Gravimetrically, total FA (mg/g DM) increased significantly in fed stage I and II larvae as did the

TABLE 1.

The dry mass (DM) per individual (mg), lipid content (mg/g DM and μ g/individual) and size (mean \pm SD) of phyllsoma larvae of the western rock lobster, *Panulirus cygnus*, during feeding and starvation.

	Mass/Ind (mg)*	Lipid (mg/g DM)*	Lipid (μ g/ind)*	Size (mm)†
Hatch (1)	0.079 \pm 0.004 st	42.9 \pm 1.2 ^{ab}	3.4 \pm 0.1 ^d	1.76 \pm 0.02 ^d
Stage I middle fed	0.092 \pm 0.007 ^{ab}	45.5 \pm 1.4 ^{ab}	4.2 \pm 0.2 ^{ab}	
Stage I middle starved	0.056 \pm 0.004 ^l	33.3 \pm 4.3 ^b	1.9 \pm 0.1 ^c	
Stage II beginning	0.099 \pm 0.001 ^{bc}	48.0 \pm 5.8 ^a	4.8 \pm 0.5 ^b	
Hatch (2)	0.081 \pm 0.006 ^a	53.5 \pm 5.7 ^{ac}	4.3 \pm 0.2 ^{ab}	1.81 \pm 0.02 ^b
Stage II beginning	0.110 \pm 0.004 ^c	68.9 \pm 7.2 ^c	7.6 \pm 0.9 ^c	
Stage II middle fed	0.147 \pm 0.007 ^d	100.4 \pm 6.8 ^d	14.8 \pm 0.4 ^d	
Stage II middle starved	0.098 \pm 0.004 ^{bc}	34.6 \pm 4.5 ^b	3.4 \pm 0.4 ^c	
Stage III beginning	0.222 \pm 0.015 ^c	66.0 \pm 5.9 ^c	14.6 \pm 0.4 ^d	

Hatch 1 and 2 are from different females. Data, within a column, not sharing a common superscript are significantly different ($P < 0.05$).

* $n = 3$.

† $n = 20$; measured from the anterior margin of the cephalic shield to the posterior of the abdomen.

TABLE 2.

Lipid class composition (mean \pm SD; $n = 3$) as percentage of total lipid in western rock lobster (*Panulirus cygnus*) phyllosoma and enriched *Artemia*.

Sample	Hydrocarbons	Wax Esters	DAGE	TAG	Free Fatty Acids	Sterols	Polar Lipids
Stage I—hatch	2.5 \pm 1.2 ^{abc}	1.3 \pm 0.5 ^{ab}	0.1 \pm 0.0	0.1 \pm 0.0 ^{ab}	0.5 \pm 0.1 ^{ab}	7.7 \pm 0.6 ^{ab}	87.8 \pm 1.4 ^{ad}
Stage I—middle fed	1.6 \pm 0.2 ^{acd}	0.3 \pm 0.1 ^{bcd}	0.1 \pm 0.1	0.1 \pm 0.0 ^{ab}	0.2 \pm 0.1 ^a	8.2 \pm 0.4 ^{ab}	89.6 \pm 0.4 ^{ab}
Stage I—middle starved	4.6 \pm 1.4 ^b	2.3 \pm 0.7 ^a	0.1 \pm 0.1	0.2 \pm 0.1 ^b	1.1 \pm 0.6 ^b	9.8 \pm 0.7 ^{ad}	82.0 \pm 2.7 ^c
Stage II—beginning	2.9 \pm 0.6 ^{ab}	1.0 \pm 0.3 ^{ad}	0.0 \pm 0.0	0.2 \pm 0.1 ^{ab}	0.4 \pm 0.4 ^a	7.3 \pm 1.4 ^{bc}	88.3 \pm 0.6 ^{abd}
Stage I—hatch	0.7 \pm 0.2 ^{cd}	0.1 \pm 0.1 ^c	0.0 \pm 0.0	0.1 \pm 0.0 ^{ab}	0.1 \pm 0.0 ^a	7.2 \pm 0.6 ^{bc}	91.8 \pm 0.3 ^{bc}
Stage II—beginning	1.1 \pm 0.8 ^{acd}	0.6 \pm 0.6 ^{bcd}	0.1 \pm 0.0	0.1 \pm 0.0 ^{ab}	0.3 \pm 0.2 ^a	6.6 \pm 0.9 ^{bc}	91.4 \pm 2.3 ^{abc}
Stage II—middle fed	0.4 \pm 0.2 ^d	0.1 \pm 0.0 ^c	0.0 \pm 0.0	0.0 \pm 0.0 ^a	0.1 \pm 0.0 ^a	5.1 \pm 1.0 ^c	94.3 \pm 0.7 ^c
Stage II—middle starved	2.6 \pm 1.2 ^{abc}	0.6 \pm 0.4 ^{bcd}	0.1 \pm 0.0	0.1 \pm 0.0 ^{ab}	0.5 \pm 0.1 ^{ab}	12.1 \pm 0.6 ^d	84.1 \pm 1.3 ^{dc}
Stage III—beginning	0.7 \pm 0.4 ^{cd}	0.2 \pm 0.1 ^{cd}	0.1 \pm 0.0	0.1 \pm 0.0 ^{ab}	0.1 \pm 0.1 ^a	7.1 \pm 0.9 ^{bc}	91.8 \pm 1.2 ^{ab}
<i>Artemia</i>	0.3 \pm 0.0	0.1 \pm 0.0	0.2 \pm 0.1	31.0 \pm 2.7	3.1 \pm 0.2	3.3 \pm 0.3	62.0 \pm 2.6

Data, within a column, not sharing a common superscript are significantly different ($\alpha = 0.05$).

TAG, triacylglycerol; DAGE, diacylglycerols ether.

major FA (Fig. 2). The sum of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in fed stage I and II larvae did not change. The sum of polyunsaturated fatty acids (PUFA) did not significantly change in fed stage I and II larvae, however there was a small increase (Table 3 and 4). Both stage I and II fed larvae showed an increase in n-3/n-6 ratio (significant in stage I) (Tables 3 and 4). The EPA/AA ratio showed a significant increase in both fed stage I and II larvae, largely due to an increase in EPA for stage I, and a decrease in AA, particularly for stage II larvae (Tables 3 and 4). The DHA/EPA ratio did not significantly change in fed stage I or II larvae.

Starved stage I and II larvae showed a gravimetric decrease in total FA, although this was only significant in stage II larvae (Tables 3 and 4). Individually, most of the major FA decreased in starved stage I larvae, although only DHA decreased significantly, and 18:0 actually increased (Fig. 2). In starved stage II larvae, all the major FA significantly decreased gravimetrically. On a percentage basis, AA increased in starved stage I and II larvae (sig-

nificant in stage II), with no significant change in EPA and DHA (Table 3 and 4). Starved stage I larvae showed no significant difference in the sum of MUFA or PUFA compared with newly hatched larvae, however SFA significantly increased (Table 3). Starved stage II larvae did not show a difference in the sum of SFA, however PUFA significantly increased and MUFA significantly decreased (Table 4). In stage I and II starved larvae, there was no change in the n-3/n-6 ratio (Tables 3 and 4). A reduction in the EPA/AA ratio was observed in starved larvae (not significant), with the DHA/EPA ratio not changing (Tables 3 and 4).

The total FA content significantly decreased in newly molted stage III larvae compared with fed stage II larvae. However, newly molted stage II larvae actually contained a higher total FA content than fed stage I larvae (Table 3 and 4).

The percentage FA composition of the *Artemia* was dominated by 16:0 (19.7%), followed by DHA (14.3%), 18:1n-9c (12.3%), EPA (10.7%), 18:1n-7c (9.8%), and 16:1n-7c (6.2%). Other FA were present at $\leq 6\%$, with AA at 2.3% (Table 3). Phyllosoma were able to

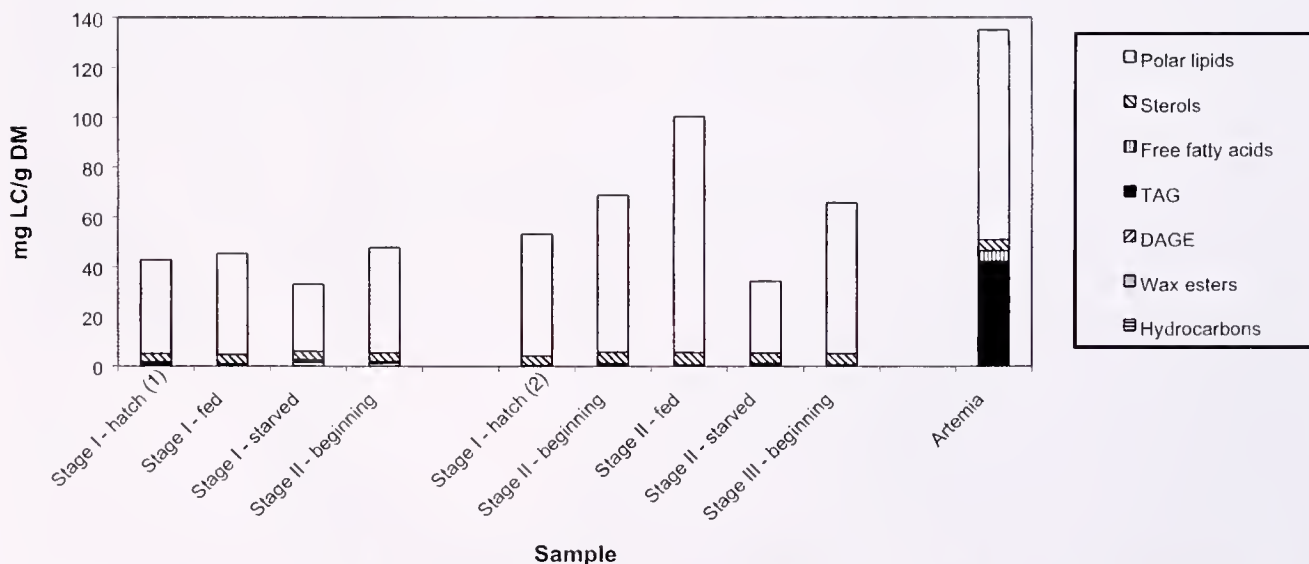


Figure 1. Lipid class content (mg/g DM) at hatch, after feeding, starvation, and molting ($n = 3$) of western rock lobster (*Panulirus cygnus*) phyllosoma and in enriched *Artemia*. LC, lipid class; TAG, triacylglycerol; DAGE, diacylglyceryl ether; DM, dry mass.

TABLE 3.

Percentage fatty acid (FA) composition and total FA levels (mg/g DM) at hatch, after feeding, starvation, and molting (mean \pm SD; $n = 3$) of western rock lobster (*Panulirus cygnus*) phyllosoma and enriched *Artemia*.

Fatty Acid	Hatch 1				
	Stage I			Stage II	
	Hatch (I)	Fed	Starved	Beginning	Artemia
14:0	0.9 \pm 0.1 ^{ab}	0.6 \pm 0.0 ^{bd}	1.0 \pm 0.3 ^{ab}	1.2 \pm 0.1 ^{ac}	5.1 \pm 0.0
16:1n-7c	2.8 \pm 0.2 ^{ac}	1.6 \pm 0.1 ^b	2.2 \pm 0.4 ^{lg}	2.6 \pm 0.1 ^{al}	6.2 \pm 0.1
16:0	12.2 \pm 0.8 ^a	10.7 \pm 0.4 ^b	12.1 \pm 0.2 ^d	13.9 \pm 0.5 ^c	19.7 \pm 0.2
17:0	0.8 \pm 0.1 ^a	0.9 \pm 0.0 ^{bd}	1.1 \pm 0.0 ^c	1.1 \pm 0.0 ^c	0.7 \pm 0.0
18:2n-6	1.1 \pm 0.2 ^a	1.4 \pm 0.0 ^{ac}	1.3 \pm 0.2 ^{ac}	1.9 \pm 0.1 ^b	1.7 \pm 0.0
18:1n-9c	11.2 \pm 0.6 ^{ad}	11.2 \pm 0.3 ^{ad}	10.7 \pm 1.0 ^{ad}	14.6 \pm 0.3 ^b	12.3 \pm 0.1
18:1n-7c	3.4 \pm 0.4 ^a	5.3 \pm 0.1 ^b	3.7 \pm 0.2 ^a	9.1 \pm 0.2 ^c	9.8 \pm 0.1
18:0	8.5 \pm 0.2 ^a	9.9 \pm 0.2 ^b	12.3 \pm 0.5 ^{cd}	11.9 \pm 0.7 ^c	5.3 \pm 0.1
18:0 FAde	2.8 \pm 0.2 ^a	2.0 \pm 0.2 ^b	3.7 \pm 0.2 ^d	1.6 \pm 0.3 ^c	0.0 \pm 0.0
20:4n-6 AA	11.2 \pm 1.0 ^{ae}	10.8 \pm 0.9 ^{abc}	12.4 \pm 0.8 ^c	8.7 \pm 0.2 ^{bc}	2.3 \pm 0.0
20:5n-3 EPA	16.7 \pm 1.1 ^a	21.3 \pm 1.0 ^b	15.6 \pm 0.7 ^{ac}	14.9 \pm 0.0 ^{ac}	10.7 \pm 0.1
20:2n-6	0.8 \pm 0.1 ^a	0.7 \pm 0.0 ^a	1.2 \pm 0.0 ^c	0.5 \pm 0.0 ^b	0.1 \pm 0.0
20:1(n-9/11)c	2.1 \pm 0.3 ^{ef}	1.3 \pm 0.0 ^b	1.8 \pm 0.1 ⁱ	1.1 \pm 0.0 ^{bc}	0.3 \pm 0.0
20:0	0.5 \pm 0.1 ^a	0.8 \pm 0.0 ^{bc}	1.4 \pm 0.1 ^c	0.9 \pm 0.0 ^b	0.2 \pm 0.0
22:5n-6	0.4 \pm 0.1 ^a	0.9 \pm 0.1 ^b	0.2 \pm 0.0 ^c	1.4 \pm 0.1 ^{cl}	4.7 \pm 0.2
22:6n-3 DHA	11.5 \pm 0.8 ^{ac}	13.0 \pm 0.4 ^a	10.3 \pm 0.5 ^{cc}	7.1 \pm 0.2 ^{bd}	14.3 \pm 0.7
22:5n-3	0.7 \pm 0.1 ^a	0.3 \pm 0.0 ^b	0.3 \pm 0.1 ^b	0.2 \pm 0.0 ^c	0.2 \pm 0.0
22:0	0.5 \pm 0.1 ^a	0.9 \pm 0.0 ^b	1.3 \pm 0.1 ^c	1.2 \pm 0.0 ^{cd}	0.7 \pm 0.0
Other	8.5 \pm 1.0	6.2 \pm 0.6	8.0 \pm 0.5	6.6 \pm 0.6	6.1 \pm 0.3
Sum SFA	24.8 \pm 1.0 ^a	24.8 \pm 0.7 ^a	30.5 \pm 0.3 ^{cd}	31.7 \pm 0.9 ^{bc}	34.0 \pm 0.3
Sum MUFA	21.7 \pm 0.7 ^{ad}	20.8 \pm 0.7 ^{ad}	19.7 \pm 1.6 ^d	28.9 \pm 0.3 ^b	30.3 \pm 0.3
Sum PUFA	45.3 \pm 1.9 ^{ac}	50.4 \pm 1.9 ^a	43.6 \pm 1.7 ^c	36.5 \pm 0.4 ^{bde}	48.1 \pm 0.5
Sum n-3	29.3 \pm 1.8 ^a	35.2 \pm 1.2 ^b	26.8 \pm 1.3 ^{ac}	23.0 \pm 0.3 ^{cd}	38.4 \pm 0.4
Sum n-6	14.9 \pm 0.6 ^{ab}	14.5 \pm 0.4 ^{ab}	15.8 \pm 0.5 ^a	13.1 \pm 0.1 ^{bc}	9.3 \pm 0.1
Ratio (n-3)/(n-6)	2.0 \pm 0.2 ^{ab}	2.4 \pm 0.3 ^c	1.7 \pm 0.1 ^a	1.8 \pm 0.0 ^a	4.1 \pm 0.0
Ratio EPA/AA	1.5 \pm 0.1 ^{acd}	2.0 \pm 0.3 ^b	1.3 \pm 0.1 ^d	1.7 \pm 0.0 ^{ab}	4.6 \pm 0.0
Ratio DHA/EPA	0.7 \pm 0.0 ⁱ	0.6 \pm 0.0 ^b	0.7 \pm 0.0 ^{ab}	0.5 \pm 0.0 ^c	1.3 \pm 0.1
Total FA (mg/g DM)	14.8 \pm 0.4 ^u	21.6 \pm 2.5 ^b	13.1 \pm 1.6 ^a	23.6 \pm 1.8 ^b	78.1 \pm 2.3

Data, within a row, not sharing a common superscript are significantly different ($P < 0.05$).

Other <1%: 12:0, i14:0, 14:1, C14PUFA, i15:0, a15:0, 15:1, 15:0, i16:0, C16PUFA, 16:1n-9c, 16:2, 16:1n-5c, 16:0 Fade, br17:1, i17:0, a17:0, 17:1, 18:3n-6, i18:0, 18:4n-3, 18:3n-3, 18:1n-5c, C19PUFA, i19:0, 19:1, 20:2, 20:3n-6, 20:2NMI, 20:4n-3 20:1n-7c, C20Falde, C21PUFA, 21:0, 22:4n-6, 22:1, C28PUFA.

AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; NMI, non-methylene interrupted; Fade, fatty aldehyde; DM, dry mass.

accumulate AA and EPA at percentage levels above that in *Artemia*, but not DHA, which was always lower in phyllosoma.

DISCUSSION

Changes in Phyllosoma Dry Mass and Lipid Content

In both larval stages, there were increases in the DM and in the amount of lipid in fed larvae, with decreases in starved larvae. Changes were more significant in stage II larvae (Table 1). Lipids are used during starvation in other crustaceans (Virtue et al. 1993), and previous research has found lipid a key component in larval stages of spiny lobsters (Jeffs et al. 1999, Phleger et al. 2001). However, lipid only accounted for 6.5% of the decrease in DM in starved stage I larvae whereas this increased to 35.0% for stage II larvae. Also, lipid only accounted for 6.2% of the increase in DM of fed stage I larvae, increasing to 19.7% in stage II larvae. This indicates that lipid was not the major nutrient catabolized during starvation or accumulated by the feeding phyllosoma. Smith et al.

(2003) suggested that protein, and to a lesser extent carbohydrate, would have been preferentially catabolized during starvation of stage I *J. edwardsii* phyllosoma in which lipid accounted for 17.8% in the loss of DM. This higher value in *J. edwardsii* may be related to the larger size of stage I larvae compared with *P. cygnus*. D'Souza (1998) found starved *Penaeus japonicus* and *Penaeus monodon* larvae exhibited gravimetric decreases in both lipid and protein.

Larvae molting to successive stages showed an increase in DM, with stage II larvae molting to stage III also showing a reduction in the lipid content (Table 1), possibly used during the molt. Smith et al. (2003) suggested that the increase in lipid accumulation seen in fed *J. edwardsii* phyllosoma might be used during the subsequent moult, as found with American lobster (*Homarus americanus*) larvae (Sasaki 1984). A decrease was not seen in newly molted stage II *P. cygnus* larvae compared with fed stage I, the lipid level was actually higher in the newly molted stage II larvae (Table 1). This may be attributable to a large variation in the

TABLE 4.

Percentage fatty acid (FA) composition and total FA levels (mg/g DM) at hatch, after feeding, starvation, and molting (mean \pm SD; $n = 3$) of western rock lobster (*Panulirus cygnus*) phyllosoma.

Fatty acid	Hatch 2				
	Stage I	Stage II			Stage III
	Hatch (2)	Beginning	Fed	Starved	Beginning
14:0	0.4 \pm 0.0 ^d	1.2 \pm 0.2 ^{ac}	1.4 \pm 0.1 ^c	1.1 \pm 0.2 ^{ac}	1.2 \pm 0.1 ^{ac}
16:1n-7c	2.7 \pm 0.0 ^a	3.2 \pm 0.0 ^{cd}	3.7 \pm 0.1 ^{de}	1.8 \pm 0.1 ^{bg}	3.8 \pm 0.2 ^c
16:0	13.7 \pm 0.0 ^c	14.0 \pm 0.2 ^c	15.2 \pm 0.3 ^d	12.6 \pm 0.1 ^a	14.6 \pm 0.3 ^{cd}
17:0	1.2 \pm 0.0 ^a	0.9 \pm 0.0 ^{bd}	0.9 \pm 0.0 ^{ib}	1.0 \pm 0.0 ^{bc}	1.1 \pm 0.0 ^{cd}
18:2n-6	1.2 \pm 0.0 ^{ac}	1.9 \pm 0.0 ^b	1.9 \pm 0.0 ^b	1.5 \pm 0.1 ^c	1.9 \pm 0.0 ^b
18:1n-9c	12.4 \pm 0.0 ^{ac}	13.8 \pm 0.1 ^{bc}	14.5 \pm 0.1 ^b	10.6 \pm 0.2 ^d	13.6 \pm 0.2 ^{bc}
18:1n-7c	4.5 \pm 0.0 ^d	10.4 \pm 0.5 ^e	12.0 \pm 0.1 ^f	8.2 \pm 0.1 ^g	12.3 \pm 0.2 ^f
18:0	9.8 \pm 0.1 ^{ab}	11.8 \pm 0.1 ^c	11.7 \pm 0.1 ^c	13.9 \pm 0.1 ^d	12.8 \pm 0.2 ^{cd}
18:0 Fade	3.8 \pm 0.0 ^d	1.5 \pm 0.1 ^c	0.8 \pm 0.0 ^e	2.3 \pm 0.2 ^{ab}	1.0 \pm 0.0 ^e
20:4n-6 AA	9.9 \pm 0.0 ^{ab}	7.4 \pm 0.3 ^{cd}	5.8 \pm 0.0 ^d	9.8 \pm 0.2 ^{ab}	6.0 \pm 0.1 ^d
20:5n-3 EPA	13.7 \pm 0.1 ^c	14.9 \pm 0.1 ^{ac}	14.2 \pm 0.2 ^{ac}	16.4 \pm 0.1 ^{ac}	14.6 \pm 0.2 ^{ac}
20:2n-6	1.3 \pm 0.0 ^c	0.5 \pm 0.0 ^b	0.3 \pm 0.0 ^d	0.7 \pm 0.1 ^a	0.3 \pm 0.0 ^d
20:1(n-9/11)c	2.3 \pm 0.0 ^a	1.0 \pm 0.0 ^{cd}	0.8 \pm 0.0 ^{de}	1.0 \pm 0.0 ^{cd}	0.7 \pm 0.0 ^e
20:0	0.7 \pm 0.0 ^{bc}	0.8 \pm 0.1 ^{bc}	0.6 \pm 0.0 ^{ad}	1.1 \pm 0.0 ^f	0.7 \pm 0.0 ^{cd}
22:5n-6	0.4 \pm 0.0 ^a	1.6 \pm 0.1 ^c	1.9 \pm 0.1 ^d	1.2 \pm 0.0 ^f	1.6 \pm 0.1 ^c
22:6n-3 DHA	10.5 \pm 0.1 ^c	7.9 \pm 0.2 ^{bd}	7.8 \pm 0.3 ^{bd}	8.6 \pm 0.1 ^{bc}	7.0 \pm 0.3 ^d
22:5n-3	1.1 \pm 0.0 ^d	0.2 \pm 0.0 ^{ce}	0.2 \pm 0.0 ^{ce}	0.1 \pm 0.0 ^f	0.1 \pm 0.0 ^{ef}
22:0	0.9 \pm 0.0 ^b	1.2 \pm 0.1 ^{cd}	1.0 \pm 0.0 ^{bd}	1.8 \pm 0.0 ^e	1.2 \pm 0.01 ^d
Other	10.1 \pm 0.0	6.4 \pm 0.1	5.8 \pm 0.1	7.3 \pm 0.7	6.1 \pm 0.2
Sum SFA	28.2 \pm 0.1 ^d	31.6 \pm 0.3 ^{bc}	32.5 \pm 0.4 ^{bc}	33.0 \pm 0.6 ^b	33.2 \pm 0.4 ^b
Sum MUFA	24.7 \pm 0.1 ^c	29.9 \pm 0.5 ^{bc}	32.5 \pm 0.2 ^c	22.8 \pm 0.3 ^{ac}	31.9 \pm 0.5 ^c
Sum PUFA	41.5 \pm 0.2 ^{bc}	26.0 \pm 0.2 ^{de}	33.6 \pm 0.7 ^e	40.2 \pm 0.2 ^{bcd}	32.9 \pm 0.8 ^e
Sum n-3	25.9 \pm 0.1 ^{acd}	23.7 \pm 0.2 ^{cd}	22.9 \pm 0.5 ^{cd}	25.8 \pm 0.2 ^{acd}	22.3 \pm 0.6 ^d
Sum n-6	14.3 \pm 0.0 ^{ab}	11.9 \pm 0.2 ^{cd}	10.5 \pm 0.1 ^{de}	13.6 \pm 0.1 ^{bc}	10.2 \pm 0.2 ^e
Ratio (n-3)/(n-6)	1.8 \pm 0.0 ^a	2.0 \pm 0.0 ^{ab}	2.2 \pm 0.0 ^{bc}	1.9 \pm 0.0 ^{ab}	2.2 \pm 0.0 ^{bc}
Ratio EPA/AA	1.4 \pm 0.0 ^{cd}	2.0 \pm 0.1 ^b	2.4 \pm 0.0 ^c	1.7 \pm 0.0 ^{abc}	2.4 \pm 0.0 ^c
Ratio DHA/EPA	0.8 \pm 0.0 ^c	0.5 \pm 0.0 ^{cd}	0.6 \pm 0.0 ^d	0.5 \pm 0.0 ^{cd}	0.5 \pm 0.0 ^c
Total FA (mg/g DM)	27.4 \pm 3.2 ^{bc}	33.1 \pm 0.9 ^c	57.8 \pm 7.5 ^d	14.0 \pm 0.7 ^a	33.7 \pm 2.9 ^c

Data, within a row, not sharing a common superscript are significantly different ($P < 0.05$).

Other <1%: 12:0, i14:0, 14:1, C14PUFA, i15:0, a15:0, 15:1, 15:0, i16:0, C16PUFA, 16:1n-9c, 16:2, 16:1n-5c, 16:0 Fade, br17:1, i17:0, a17:0, 17:1, 18:3n-6, i18:0, 18:4n-3, 18:3n-3, 18:1n-5c, C19PUFA, i19:0, 19:1, 20:2, 20:3n-6, 20:2NMI, 20:4n-3 20:1n-7c, C20Falde, C21PUFA, 21:0, 22:4n-6, 22:1, C28PUFA.

AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; NMI, non-methylene interrupted; Fade, fatty aldehyde; DM, dry mass.

molting period of the larvae. Larvae from the first hatch took 3 days to all molt to stage II before a sample was taken. Therefore, many of the sampled larvae may have started to accumulate lipid.

Lipid Class

Variation Between Hatches and Species

Newly hatched larvae from the second hatch had elevated concentrations of lipid (PL and total FA) compared with larvae from the first hatch, with larvae from the second hatch also being significantly larger (Tables 1, 2, 3, and 4). This may be attributable to different temperatures during egg incubation. Both females extruded eggs at 25°C, however eggs from the first hatch were incubated in water at 22°C whereas those from the second hatch were at 19°C. Smith et al. (2002) found newly hatched *J. edwardsii* phyllosoma from warmer water were significantly smaller. The warmer incubation temperature may have resulted in more energy being used for metabolism and less for development of the em-

bryo. This difference at hatching between batches of *P. cygnus* larvae had become significant at the molt to stage II (Table 1). Larvae from the second hatch showed significantly higher lipid content (PL and total FA), suggesting the condition of larvae at hatch affected their condition into stage II, and possibly for further culture.

The absence or very low levels of TAG in phyllosoma samples has been a common feature found in studies so far (Nelson et al. 2003, Phleger et al. 2001), including this study (Table 2). TAG is generally the most common storage lipid in animals, used as a short-term energy reserve, and it is generally catabolized primarily during starvation (Koven et al. 1989, Olsen 1998, Phleger et al. 2001). This is in contrast to PL, which plays an important structural role and is usually preferentially conserved (Koven et al. 1989). PL was the major LC in *P. cygnus* larvae comprising 82.0–94.3% of the total lipid from all samples (Table 2), as found with *J. edwardsii* phyllosoma (Nelson et al. 2003, Phleger et al. 2001). In this study, the increase found in lipid (mg/g DM) in both stages

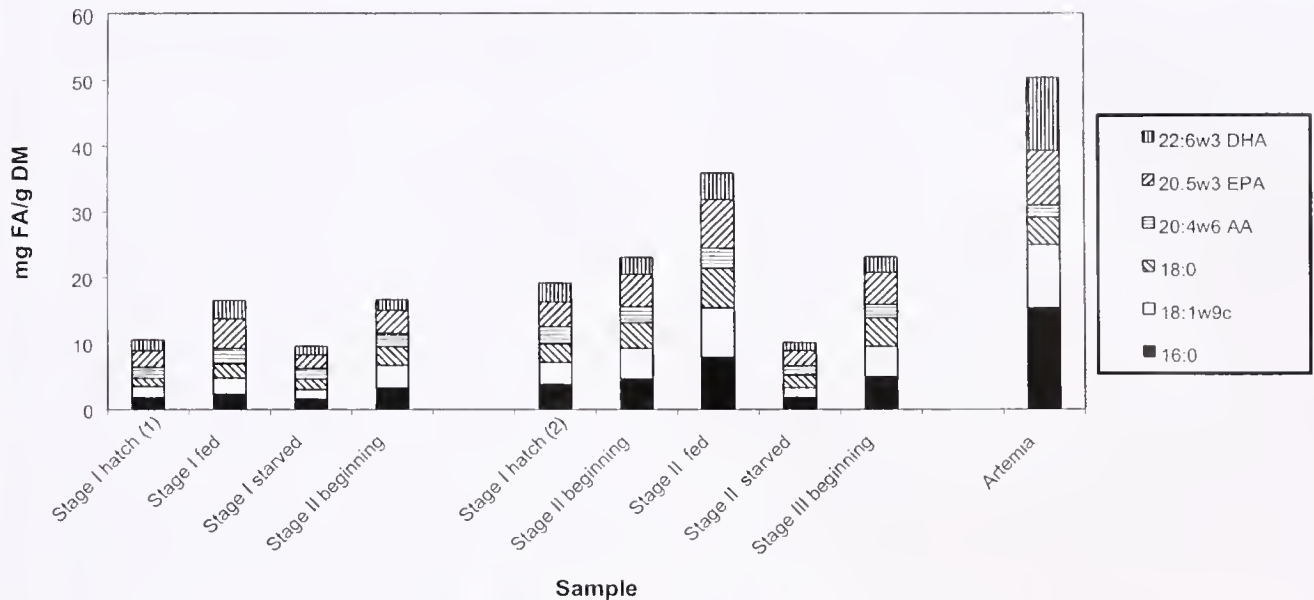


Figure 2. The content of major fatty acids (mg/g DM) at hatch, after feeding, starvation, and molting ($n = 3$) of western rock lobster (*Panulirus cygnus*) phyllosoma and in enriched *Artemia*. FA, fatty acid; DM, dry mass; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

of fed *P. cygnus* larvae corresponded almost completely to the increase in PL (mg/g DM). The use of PL as the dominant storage medium in the puerulus stage of rock lobsters is unlike many other marine taxa, including crustacea, which tend to use TAG and WE (Jeffs et al. 2001, Sasaki 1984). Jeffs et al. (2001) found PL reserves were primarily used during the puerulus stage of *J. edwardsii* and suggested the use of PL as a storage medium in the puerulus may be related to its characteristic transparency, an important feature of this nektonic stage that is highly vulnerable to pelagic visual predators. Jeffs et al. (2001) suggested pueruli avoid storing neutral lipid because of its opaque nature. The prevalence of PL as the major LC in phyllosoma larvae may also be due to their transparency, which could provide protection during their long larval ocean phase.

PL was also the major LC used during starvation and was significantly reduced in starved larvae from both stages (Table 2, Fig. 1). Lipid was found to be the primary energy source used during the nonfeeding puerulus stage and PL was also the major LC used (Jeffs et al. 1999, Jeffs et al. 2001). PL was also the only LC to be depleted when stage II larvae molted to stage III, with a significant gravimetric reduction (Fig. 1).

ST (mainly cholesterol) was the next most abundant LC in the phyllosoma samples (Table 2). PL, together with cholesterol and sphingolipids, are omnipresent components of cell membranes and are therefore both structurally and functionally important (Olsen, 1998). Unlike PL, relative amounts of ST increased in starved larvae, with no significant decrease in gravimetric amount (mg/g DW) (Table 2, Fig. 1). This retention may indicate the importance of ST, as ST are used not only in membranes but also in the transport of lipids (Ackman 1998).

Phyllosoma were able to maintain a higher percentage of PL and ST than found in their diet (Table 2). *Artemia* contained lower levels of PL (62.0%) and ST (3.3%), with higher levels of TAG (31.0%) and FFA (3.1%) than phyllosoma (Table 2). It appears PL and ST are important nutrients to the phyllosoma, hence their

accumulation at levels (%) above that found in their food. However, TAG is almost absent (<0.2%) in the phyllosoma samples (Table 2).

The phyllosoma lipid was around 90% PL, and comparatively the PL in *Artemia* was much lower (60%). TAG makes up a large portion in *Artemia*, and it is possible that phyllosoma cannot absorb or accumulate the TAG as efficiently as PL to gain nutrients.

Fatty Acids

The major FA and their profiles in newly hatched *P. cygnus* larvae (Tables 3 and 4) are similar to those reported for newly hatched *J. edwardsii* phyllosoma (14.2–14.6% 16:0, 12.6–13.3% 18:1n-9c, 6.9–7.1% 18:0, 11.6–11.9% AA, 14.8–15.3% EPA, and 7.6–8.1% DHA; Phleger et al. 2001). Fed *P. cygnus* larvae also showed a similar relative contribution from the major FA, with all the major FA significantly increasing gravimetrically. However, the relative levels were different to those found in their diet. The FA distribution of animals is believed to be primarily determined by the composition of their dietary FA (Napolitano 1998, Olsen 1998). In comparison, for the current study 16:0 and DHA were at lower relative levels than found in the *Artemia*, with 18:0, AA, and EPA at levels above that found in the *Artemia*, possibly indicating a preferential accumulation of these FA. DHA showed a continual reduction in relative contribution as the phyllosoma developed (fed and molting samples). This may suggest that the DHA was not in a form that the phyllosoma could absorb and use or was not at high enough levels. Nichols et al. (2001) examined a wide range of potential prey items for *J. edwardsii* phyllosoma and found that DHA was in average 26%, markedly higher than that in the *Artemia*. The prey also contained on average 77% PL, again far greater than occurring in *Artemia*. AA also declined throughout development; however the level of AA in the *Artemia* was always much lower than found in the phyllosoma.

The increase in the EPA/AA ratio in fed larvae suggests a higher accumulation of EPA in fed larvae, however, starved larvae

did exhibit a reduced ratio (not significant) suggesting more AA was conserved during starvation (Table 3 and 4). The DHA/EPA ratio did not show major changes in fed or starved stage I or II larvae, although some were statistically significant, suggesting both FA were used/retained at a similar rate (Tables 3 and 4).

To formulate a diet that meets the essential fatty acid (EFA) requirements of a given species, it is necessary to know the dominant EFA series for this species (Corraze 2001). Experiments have found that during starvation larvae conserve important FA, with starvation suggested as one way to determine nutritional requirements of larvae (Koven et al. 1989, Olsen 1998). Starved *P. cygnus* larvae showed a gravimetric decrease in total FA, however, a number of individual FA showed no change or assumed a greater ranking in the FA profile, possibly signifying a greater degree of importance. The FA changes were however dependent on the stage tested. The relative contribution of AA increased in starved stage I and II larvae, although the change in starved stage I larvae was not significant. EPA and DHA showed increases in stage II larvae, however, in starved stage I larvae there was a small decrease. D'Souza (1998) found PUFA (AA, EPA and DHA) were conserved in starved *Penaeus* larvae and suggested this was most likely due to their primarily structural role.

Starved stage I larvae showed a significant increase in SFA, with no change in MUFA or PUFA. However, in starved stage II larvae, MUFA significantly decreased whereas PUFA significantly increased, possibly indicating a preferential use of MUFA during starvation and a sparing or retention of PUFA. Kattner et al. (1994) found the FA profile of larval caridean shrimp also showed major

differences dependent on stage and larval sample. The variation in results in the present study suggest a response for *P. cygnus* which is dependent on the stage of the larvae.

CONCLUSIONS

Although lipid was not a major component of the larvae and was not the major nutrient accumulated in fed larvae or used in starved larvae, lipid became a greater component in stage II larvae compared with stage I larvae, suggesting that lipid likely assumes a greater importance as larvae progress through developmental stages. Further studies should also assess the importance of other nutrients (i.e., protein and carbohydrate) in phyllosoma development. The LC profile of the phyllosoma samples was dominated by PL. PL was also the main LC used during starvation and accounted for almost the entire increase in lipid in fed larvae. ST was the next most abundant LC and showed an increase in relative contribution in starved larvae, its retention possibly related to its important structural/function role. *Artemia* had high levels of TAG, however phyllosoma never accumulated TAG. The FA changes occurring in larvae appeared to be stage dependent, however PUFA, such as AA, EPA and DHA, were generally conserved (particularly in stage II larvae). Results from the current study provide information that will assist in formulating diets for phyllosoma culture. Larval feeds containing lipids rich in EFA (EPA, DHA and AA) and in a form allowing them to synthesize PL may be more suitable for future research with *P. cygnus*. The *Artemia*, as enriched in this study, do not meet these criteria, with current work to focus on enriching *Artemia* to make them reflect the perceived profile.

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ABSTRACTS OF TECHNICAL PAPERS

Presented at the 96th Annual Meeting

NATIONAL SHELLFISHERIES ASSOCIATION

Honolulu, Hawaii

March 1–5, 2004

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PREDICTING THE EFFECTS OF A CHANGING COASTAL LANDSCAPE ON LARGE-SCALE CLAM AQUACULTURE. Gretchen L. Arnold, Mark W. Luckenbach, Harry V. Wang, and Jian Shen, Eastern Shore Laboratory Virginia Institute of Marine Science College of William and Mary Wachapreague, VA 23480, USA. E-mail: gretchen@vims.edu

Hard clam (*Mercenaria mercenaria*) production in the lower Chesapeake Bay, Virginia, USA has increased dramatically in the last decade. Cultured clams are densely planted in shallow zones adjacent to land, often in small embayments or tidal creeks, where land use, water quality, and bivalve production are closely linked through nutrient and phytoplankton dynamics. Meanwhile, the coastal agricultural and forested lands in this region are under increasing development pressure. This changing land use has the potential to impact clam aquaculture by changing loadings of nutrients, bacteria, suspended sediments, and fresh water delivered to estuaries, resulting in altering primary production.

In an effort to examine the links between land use, water quality, and hard clam aquaculture production in these systems, we have initiated a large-scale modeling and field verification effort. The project is linking several sub-models: (1) a basin-level watershed loading sub-model; (2) a physical transport-based water quality sub-model; (3) a sediment suspension/resuspension sub-model; (4) a clam physiology sub-model; and (5) an aquaculture production sub-model into an integrated modeling framework. The framework as a whole will be calibrated and verified by field data and then used to estimate carrying capacity under current conditions and provide predictions under alternative development scenarios. This project is being conducted in Cherrystone Inlet, a tidal creek in the lower Chesapeake Bay with an estimated standing stock of 80 million cultured clams.

In this portion of the study we examine how specific land-use management decisions would affect estuarine water quality and exploitation carrying capacity for clam aquaculture in Cherrystone Inlet. The results will provide local government with a basis for evaluating how effects of zoning decisions may affect the aquaculture industry, provide the aquaculture industry with an estimate of exploitation carrying-capacity for the system, and begin to describe the ecological interactions between large-scale clam aquaculture, primary production, and land use.

MITOCHONDRIAL GENETIC VARIABILITY OF CULTURED VERSUS WILD NORTHERN HARD CLAMS *MERCENARIA MERCENARIA*. Patrick Baker, Claudia Rocha, Brian Bowen, Paoleto Solo, Luiz Rocha, and Leslie Sturmer, Fisheries and Aquatic Sciences, University of Florida, Gainesville, FL 32611, USA. E-mail: pbaker@mail.ifas.ufl.edu

Aquaculture of northern hard clams, *Mercenaria mercenaria*, is a significant industry in Florida, USA. Hard clams are grown to market size in shallow coastal plots, but seed stocks are produced

in commercial hatcheries. Hatchery stocks are usually selected for the *notata* strain, a desirable shell color variation with no other known performance advantages. Concern was raised that hatchery-breeding practices could reduce genetic variability, and hence, potentially reduce performance, compared with wild stocks. To evaluate this concern, mitochondrial cytochrome oxidase I (COI) sequences (542 bp) were generated from five Florida hatchery stocks and in clams escaped from aquaculture in west Florida. The mtDNA diversity in captive strains was compared to wild stocks from east Florida, Georgia, and New York. *M. campechiensis*, a morphologically similar, sympatric congener, sometimes occurred in samples but showed about 1%–1.5% Tamura-Nei genetic distance from *M. mercenaria* and was removed prior to statistical analysis. High haplotype diversity was found in COI sequences for all wild populations and some hatchery stocks (Table 1). Some hatchery stocks still showed levels of genetic variability within the typical range for a marine bivalve, but two had significantly reduced haplotype diversity. Two hatcheries also had apparent contamination with *M. campechiensis*. These findings indicate reduced diversity in some hatcheries, but others managed to avoid depletion of genetic resources. Further surveys with microsatellite DNA are mandated to assess inbreeding and the comparative performance of breeding stocks.

TABLE 1.
Genetic variability in wild and hatchery stocks of
Mercenaria mercenaria.

Locality/stock	Haplotype diversity	Nucleotide diversity
Southeast Florida Wild	0.93 ± 0.12	0.006 ± 0.004
Northeast Florida Wild	0.75 ± 0.09	0.005 ± 0.003
Georgia Wild	0.84 ± 0.09	0.005 ± 0.003
New York Wild	0.80 ± 0.11	0.004 ± 0.003
Hatchery I	0.78 ± 0.15	0.004 ± 0.003
Hatchery II	0.33 ± 0.21	0.0006 ± 0.0008
Hatchery III	0.50 ± 0.05	0.003 ± 0.002
Hatchery IV	0.75 ± 0.14	0.005 ± 0.003
West Florida Culture Escape	0.62 ± 0.16	0.003 ± 0.002

SELECTIVE BREEDING TO IMPROVE RESISTANCE AGAINST SUMMER MORTALITY IN THE PACIFIC OYSTER *CRASSOSTREA GIGAS*: RESULTS AFTER 3 GENERATIONS. Pierre Boudry, Lionel Dégremont, Edouard Bédier, and Jean François Samain, Laboratoire IFREMER de Génétique et Pathologie, Ronce les Bains, 17390 La Tremblade, France. E-mail: Pierre.Boudry@ifremer.fr

Summer mortality of adults and juveniles has been reported in the Pacific oyster, *Crassostrea gigas*, for many years and in several countries. The French multidisciplinary program "Morest" aims to investigate the causes of the summer mortality in *Crassostrea gigas*. Within this program, we designed multi-site field

experiments to assess to what extent genetic variability exists for summer mortality in French populations of *C. gigas* and to determine whether selective breeding could improve survival.

The first generation was based on 3 sets of bi-parental families, following a nested half-sib crossing design. Progenies (G1: 17 half-sib families (HSF)) were placed in three sites along French coasts in February, March, and April 2001 and reared following usual practices until October 2001. A large variation among HSF was observed (Fig. 1). Heritability estimates for survival were very high (mean $h^2 = 0.81 \pm 0.29$). In 2002, 3 "good" HSF and 3 "bad" HSF were selected on their survival performances to constitute a second generation (G2) by divergent selection. Additionally, these were also crossed to make inbred families. Both inbred and outbred families, reared similarly to the first generation, showed a good response to selection (realized heritabilities >0.7) (Fig. 2). No significant effect was observed on growth performance of the selected families. In 2003, a third generation of inbred and outbred families was produced to confirm the results obtained in 2002. Globally, our results indicate that selective breeding programs can efficiently improve survival of juvenile oysters.

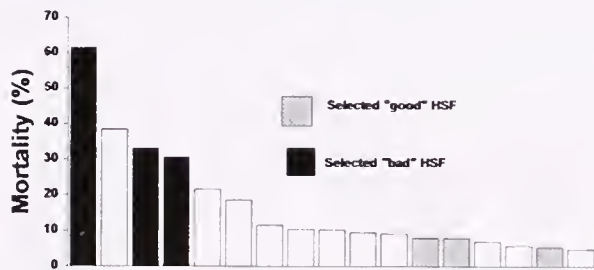


Figure 1. Variation of mean mortality among HSF in the G1 ($P < 0.001$).

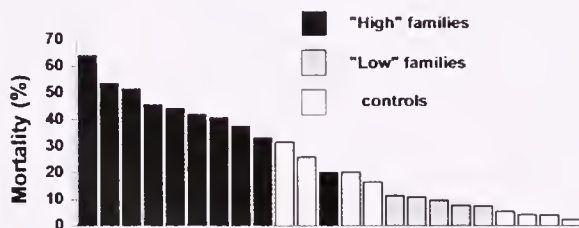


Figure 2. Variation of mean mortality the Families in the G2 (divergent selection) in Brittany ($P < 0.001$).

FRESHWATER MOLLUSCS OF THE WESTERN UNITED STATES: WHERE ARE WE TODAY, AND WHERE ARE WE GOING? Jayne Brim Box and Jeff Kershner, USDA Forest Service Rocky Mountain Research Station, 860 N 1200 E., Logan, UT 84321, USA. E-mail: brimbox@comcast.net

The western states contain at least six endemic mussel species, and many endemic snail species. Records of western freshwater mollusks date from the mid 1800s, but there is a dearth of current

information on the distribution and abundance of western freshwater mollusks, in part because a comprehensive survey throughout their distributional ranges has not been done. There is also confusion regarding the taxonomic status of western species, and the exact number of valid species that occur in the region is not clear. Although several western states recognize that mollusk populations are declining, conservation and recovery efforts are hampered by the lack of basic information on western mollusk genetics, zoogeography, systematics, and host fish. In addition, the conservation status for most western mollusks is unknown. The objectives of our work were to produce a database of all western freshwater mollusk species and their historical distributions, produce a synonymy of western freshwater mollusks that includes all previously described western species, compile a georeferenced distributional database for all western mollusk mussels, and to conduct additional field surveys, host fish analyses, and genetic work. Data on historical occurrences, habitat, life history, and other information on western mollusks were entered into a relational database. Distributional data were georeferenced, and special attention was given to nomenclature issues in order to determine whether some of the previously described western species deserve species-level status. To date, approximately 1,000 records of unionid mussels and 1,400 records of freshwater gastropods have been compiled from over 180 publications and museum records. These data were augmented by current field studies conducted in five western states. We also conducted an intensive genetic survey of western mussel populations to describe patterns of phylogeny and gene flow. The results of our studies raise intriguing questions about taxonomy, reproduction, gene flow, and host fish requirements in western mollusk species.

BIOCHEMICAL CHANGES IN JUVENILE HARD CLAMS *MERCENARIA MERCENARIA* IN RESPONSE TO HEAT AND COLD SHOCK. Nicole T. Brun, Neil W. Ross, V. Monica Bricelj, Alaina R. Boyd, and Thomas H. MacRae, Dalhousie University and the National Research Council, Institute for Marine Biosciences, Halifax, Nova Scotia Canada B3H 3Z1. E-mail: Nicole.Brun@nrc-cnrc.gc.ca

In Atlantic Canada, there has been a continuing interest in the development of hard clam *Mercenaria mercenaria* aquaculture. There are, however, limitations and challenges for such an industry in this region, because it represents the northern limit of the distributional range of *M. mercenaria*. Recently, juvenile hard clams have suffered heavy mortalities during over-wintering in Atlantic Canada. An important factor, thought to be associated with the latter, is the health status of the animals on entering the over-wintering period, and/or a combination of acute and chronic thermal stress experienced during the winter months, when the animals are unable to feed. Because there is very limited information on the physiology of juvenile *M. mercenaria* at low temperatures, espe-

cially below 12 °C, the exact causes and mechanisms of the observed over-wintering mortalities and its prevention deserve further attention.

Heat shock proteins (HSPs) are ubiquitous, highly conserved, and have been shown to increase on exposure to various stressors, including temperature. HSP expression has been well characterized in mussels, but limited information is available for other bivalves, such as hard clams. Cold shock Stress protein response (SPR) has not been previously investigated in bivalves. Characterization of the SPR to acute temperature shock may have application in acquired thermotolerance of hard clams transferred from hatchery to field grow-out sites.

Experiments were conducted to examine whether, and to what extent rapid temperature increases and/or drops induce a SPR in hard clams, and to examine HSP expression in hard clams under laboratory-simulated over-wintering conditions. The SPR to acute heat shock (20 °C to 30 °C for 3 h) and acute cold shock (20 °C to 3 °C for 3 h), determined by HSP70, was examined in juvenile *M. mercenaria* (Fig. 1). HSP70 decreased significantly during heat shock, but slowly increased during recovery period and exceeded control levels after 24 h. Interestingly, cold shock also modulated HSP70 expression. HSP70 increased significantly, with levels still rising after 24-h recovery. Experiments designed to simulate over-wintering conditions in juvenile hard clams are ongoing, and preliminary results reveal no increase in HSP70 expression clams subjected to a 3-h cold shock from 12 °C to 1 °C. The results of this study are currently being used to validate other biochemical indicators, such as proteases, and preliminary results on the latter will be presented.

IDENTIFICATION OF A HERPES-LIKE VIRUS IN PACIFIC OYSTERS FROM TOMALES BAY, CALIFORNIA. Colleen A. Burge, Robyn M. Estes, Nancy A. Stokes, John S. Hargrove, Bruce J. Barber, Ralph A. Elston, Eugene M. Bureson, Kimberly S. Reece, and Carolyn S. Friedman, School of Aquatic and Fishery Sciences, University of Washington, 1122 NE Boat Street, Seattle, WA 98195, USA. E-mail: cab3@u.washington.edu

Beginning in 1993, large-scale losses of Pacific oyster seed have been observed in Tomales Bay, California. These losses have been attributed to multiple environmental stressors including elevated water temperatures and phytoplankton blooms. An oyster herpes-like virus infecting various species of oyster larvae and juveniles was first described in French and New Zealand hatcheries in 1992 and was associated with high mortality rates. Given the timing and scope of the Pacific oyster seed mortalities in conjunction with elevated seawater temperatures associated with these declines, we conducted a preliminary assessment of the contribution of this infectious agent in these losses. Due to the importance of this viral agent to international trade, we examined the host and geographic distribution of this possible oyster pathogen in many locations in the United States. Although only recently observed in Pacific oysters, we sampled eastern (*Crassostrea virginica*), pacific (*C. gigas*) and Suminoe (*C. ariakensis*) oysters for presence of the herpes virus using molecular and histologic methods from Maine, Virginia, Florida, Louisiana, California (Tomales Bay), Oregon, and Washington. A polymerase chain reaction (PCR) method based on published sequence information was validated and optimized for use in these studies. Positive control material was kindly provided to us by collaborators from IFREMER (T. Renault).

Only Pacific oysters sampled from Tomales Bay, CA in the summer of 2002 were infected by the herpes-like virus as evidenced by PCR analysis. However, no clear microscopic confirmation of herpes-like nuclear inclusions has been observed to date. Sequence analysis determined that the virus is nearly identical to the virus observed in oysters and clams reared in France. These data suggest that further studies be warranted. We are in the process of further examining the influence of this virus on oyster survival in Tomales Bay, California.

LINE-CROSSING WITHIN AND AMONG FIVE COMMERCIAL STRAINS OF HARD CLAMS, *MERCENARIA MERCENARIA*: GROWTH, SURVIVAL, AND SUSCEPTIBILITY TO QPX DISEASE AT DIFFERING SALINITIES. Mark D. Camara, current address: Hatfield Marine Science Center, USDA/ARS Aquaculture Genetics, 2030 SE Marine Science Dr., Newport, OR 97365, USA. E-mail: Mark.Camara@oregonstate.edu; Lisa M. Ragone Calvo, Ryan B. Carnegie, and Standish K. Allen Jr., Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, Gloucester Point, VA 23062, USA.

Cultured hard clams, *Mercenaria mercenaria*, are a multi-million dollar industry in Virginia, USA. Field grow-out sites vary from full ocean salinity on the eastern shore of the Chesapeake

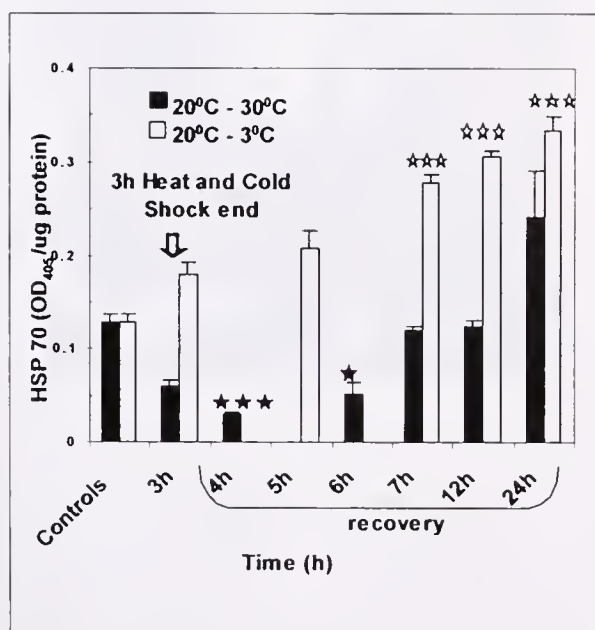


Figure 1. HSP70 expression in juvenile hard clams to acute heat (■) and cold (□) shock. $P < 0.05$.

Bay to mid-salinity estuarine sites within the bay. Quahog parasite unknown disease (QPX) has recently emerged as the causal agent of sporadic catastrophic mortalities at high salinity sites. This disease first appeared in the 1950s in New Brunswick Canada. From the late 1980s through the mid 1990s, QPX was found in Massachusetts, New Jersey, and Virginia, and first caused economically important mortalities in Virginia in 2001. QPX has the potential to threaten a large portion of the cultured clam industry on the eastern coast of the USA if the problem continues to worsen. At present, the industry depends heavily on hatchery production of essentially undomesticated genetic stocks. Relatively little is known about the suitability of stocks to different environmental conditions or their susceptibility to QPX, but previous studies indicate that stocks originating from more southern populations are more susceptible to the parasite that causes QPX.

We therefore evaluated how different commercially available clam strains perform along a salinity gradient in Virginia and the potential for enhancing growth performance and QPX resistance by outcrossing among strains. We first created all 15 possible combinations within and among five brood stock strains in the hatchery. We subsequently raised the juveniles in common conditions until they reached approximately 10 mm, at which point we split the groups for planting at five sites encompassing the range of salinities at which clams are commercially grown. We monitored their growth in the hatchery and the field and at the high salinity sites where QPX is expected to be found; we sub-sampled each group to estimate the prevalence of QPX disease within groups using standard histopathologic techniques. We then compared the growth and QPX susceptibility of groups, estimated the correlations among performance measures between life stages, compared the performance and QPX prevalence of within- and among-strain crosses, and assessed how performance measures varied among environments. We discuss the results and their implications for strain selection, hatchery-spawning procedures, and future efforts in selective breeding for superior hard clam strains.

IMPROVEMENT OF DISEASE RESISTANCE AND UNDERSTANDING OF SUMMER MORTALITY IN PACIFIC OYSTERS *CRASSOSTREA GIGAS*. Daniel Cheney, Louis Burnett, Aimee Christy, Jonathan Davis, Ralph Elston, Carolyn Friedman, Fred Griffin, Christopher Langdon, and Andrew Suhrbier, Pacific Shellfish Institute, 120 State Ave., NE #142, Olympia, WA 98501, USA. E-mail: psi@pacshell.org

Pacific oyster (*Crassostrea gigas*) summer mortalities are a recurring problem in the United States, Japan, France, and Mexico. During the 2002 to 2003 growing season, for example, farmers in Carlsbad, CA, Netarts Bay, OR, and Willapa Bay and south Puget Sound, WA reported up to a 50% loss of market-ready oysters occurring over a brief 2 to 3 week span (see Fig. 1 of oyster

cluster). Research findings indicate genetic pedigree, ploidy, culture practice, and prior exposure to environmental stress have varying influences on the expression of the summer mortality disease in Pacific oysters. Key facets of research to date include (1) the ability of oysters to survive episodes of summer mortality is in part genetically determined and perhaps can be enhanced through selective breeding of families showing high survival traits. Pedigreed lines of oysters with different genetic histories, produced by the Oregon State University Molluscan Broodstock Program (MBP), displayed markedly differential survival, yields, and growth rates during episodes of summer mortality at project study locations in California and Washington. There is a strong and consistent genetic response of families to environmental stress that is independent of when the families were produced and planted. In addition, prior inconsistent mortalities occurring across large culture plots can, at least in part, be explained by differential genetic backgrounds of the oyster crops. An ongoing western regional aquaculture center (WRAC) project is exploring an alternative approach of utilizing crossbreeding (crossing of inbred lines of oysters to produce "hybrids"). *C. gigas* heterosis ("hybrid vigor") indicates crossbreeding, in addition to traditional selection as practiced by the MBP, could improve growth and survival dramatically and quickly. Initial evaluations of hybrid lines grown out in Hood Canal, Washington in 2001 to 2002 suggest performance was exceptional.



Figure 1.

(2) Triploid and diploid oysters appear to have different optimal environments and definition of their respective requirements and response is essential for maximizing their survival and productivity. Field experiments and grower observations have demonstrated sporadic high triploid mortalities and wide variation in survival compared with diploid oysters planted at the same locations. Overall, triploid oysters grown at Puget Sound test sites in

2000 to 2002 experienced a cumulative mortality of 8%–28% above the mortality rates of diploid oysters planted in comparable plots. Tests are underway to measure the performance, survival and physiologic condition of selected strains of diploid and triploid oysters outplanted from the same parent stocks during the 2003 growing season.

ZOOSPORULATION OF PREZOOSPORANGIA DERIVED FROM IN VITRO CULTURED *PERKINSUS MARINUS* MERONTS CULTIVATED IN MEDIA SUPPLEMENTED WITH OYSTER LIPID EXTRACT. Fu-Lin E. Chu* and Eric D. Lund, Virginia Institute of Marine Science College of William and Mary Gloucester Point, VA 23062 chu@vims.edu

Four life stages of the oyster protozoan parasite, *Perkinsus marinus* have been identified and described. They include meront, prezoosporangium (hypnospores), zoosporangium, and biflagellated zoospore. Previously when tissue-associated meronts were incubated in fluid thioglycollate media (FTM) for 4-5 days, they developed to prezoosporangia, and zoosporulation (production of biflagellated zoospores) usually occurred after incubating FTM-cultured prezoosporangia in estuarine or sea water for few days. However, in contrast to what has been observed in the past, in recent years following the similar procedure, low incidence of zoosporulation without release of the zoospores has been observed in several laboratories. We hypothesize that deficiency of essential nutrients (e.g., lipids and essential fatty acids) in prezoosporangia is one of the causes of the problem. To test the hypothesis, we incubated *in vitro* cultured 21 day old meronts in alternative fluid thioglycollate media (AFTM) supplemented with oyster lipid extract and lipid-free AFTM. The concentration of lipid in the lipid-supplemented AFTM was comparable to that of oyster plasma (40 µg/ml). Prezoosporangia developed in lipid-supplemented AFTM and in non-lipid-supplement media were transferred to artificial sea water (ASW) and monitored for further development. The prezoosporangia cultured in lipid-free AFTM did not develop further. Within 24 h of transfer to ASW some prezoosporangia cultured in lipid-supplemented AFTM had developed discharge tubes. Development of cells was not synchronous, but some of the prezoosporangia exhibiting discharge tubes developed further to zoosporangia containing motile zoospores within 3 days of transfer to ASW. Free swimming zoospores were observed within 4 days of transfer to ASW. These results suggest that lipid and/or essential fatty acid storage in prezoosporangia is critical for their development to zoosporulation. Both FTM and AFTM contain limited amount of lipids and are deficient of the essential fatty acids such as arachidonic, eicosapentaenoic and docosahexaenoic acids. Presently we are determining the optimal amount of lipids required for prezoosporangia develop to zoosporulation and whether certain lipid classes or essential fatty acids are critical for such development.

DEVELOPMENT OF ANTISEPTIC PROCEDURE TO IMPROVE CULTURED PEARL FORMATION IN *PINCTADA MARGARITIFERA*. N. Cochenne-Laureau, P. Haffner, P. Levy, D. Sauhier, S. Langy, and A. Fongrouse.

Cultured black pearls from *Pinctada margaritifera* (Linnaeus) are a significant industry for French Polynesia. Pearl formation requires the inserted mantle tissue to form a complete sac around the shell nucleus and to secrete successive layers on nacre onto the bead.

Despite the relative success of this pearl formation method, substantial failures occurred. The purpose of this presentation is to study the effect of antiseptic process on mortalities from the surgery and nucleus rejection. The use of antiseptic during grafting experimentation had no significant effect on mortality and bead rejection. However, antiseptic has proven very effective in reducing the number of total bacteria isolated from pearl bag. Two dominant bacteria were isolated from *P. margaritifera* after nucleus insertion. Phenotypic and molecular characterization showed that one strain is similar to *Vibrio harveyi* and the other differs on one phenotypic character from *V. alginolitycus*. These results suggest that improvement of hygiene for all aspects of the pearl surgery really has great impact on reducing bacterial contamination. Further work is planned to confirm the possible impact of these two strains on mortality and/or nucleus rejection by experimental infection.

REVIEW ON AMINO ACID REQUIREMENT IN SHRIMP. G. Cuzon, J. Guillaume, and G. Gaxiola, Ifremer, BP 7004, Taravao, Tahiti, French Polynesia. E-mail: gcuzon@ifremer.fr

Amino acids (aa) requirement in shrimp was rapidly assessed from a qualitative point of view, using ¹⁴C acetate. Lys-Arg-His, Leu-Ile-Val, Phe, Thr, Met, and Trp. However, knowledge on quantitative requirement was hampered by technical aspects, such as leaching from purified diets, differential of absorption at midgut level between crystalline aa, and aa derived from dietary protein digestion. Among species, *P. monodon* provided the largest amount of data (Millamena et al, 1996 to 1998) with quantitative values on basic aa, branched chained aa, Phe, Met, Trp, and Thr. It was obtained with experiments on juveniles raised in clear water at low density and constant water temperature; to some extent data on larvae and PL's were included.

TABLE 1.

Iaa requirement % of protein for *P. monodon* fed 40% CP during its main phase of development with rapid growth rate.

	LYS.	ARG	HIS	LEU	ILE	VAL	THR	PHE	MET	TRP
Juveniles	5.2	5.3	2.2	4.3	2.7	3.4	3.5	3.7	2.4	0.5

Protein and aa requirement were studied with reference protein (shrimp muscle, casein, squid, clam, etc), protein requirement expressed in terms of % (relative protein requirement) is probably not so highly correlated with protein accretion as it is in vertebrates due to chitin synthesis, polyholoside containing 43% equivalent raw protein, and among aa, Thr, His, Leu, or Ala.

Amino acid requirements were assessed with defined diets described in this paper, also imbalanced diets; oxidation method and indirect oxidation methods are reported. Values obtained were evaluated and compared among species. Whole body composition for a basis of EAA pattern appeared in good correspondence (Fig. 1).

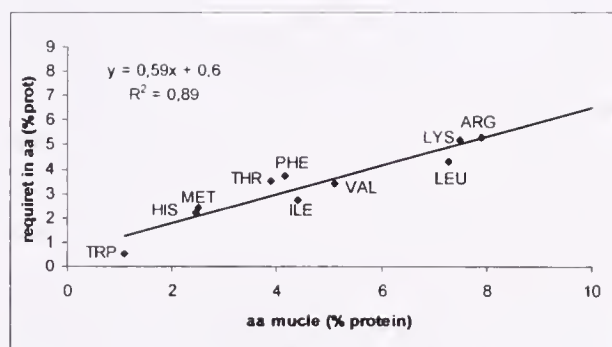


Figure 1. Correlation between shrimp muscle and aa requirement of *P. monodon*.

Several authors faced problems with leaching or absorption at midgut-level when using amino acids diets. Experiments used the technique of everted sacs to express availability of aa.

WAS HAWAII, MARCH 2004: METABOLIC RATE OF *PINCTADA MARGARITIFERA* DURING GAMETOGENESIS. G. Cuzon, C. Soyey, and G. LeMoullac, Ifremer, COP/ BP 7004 Taravao, Tahiti French Polynesia

P. margaritifera, the mother pearl is cultured extensively in French Polynesia and research on environmental rearing conditions were conducted in Tahiti (Ifremer, Service de la Perliculture, and UFP) during a multi-disciplinary program named PGRN (1993 to 1999). It led to a comprehensive approach of trophic level in lagoon waters, feeding habits, and carrying capacity.

Research for the control of spat production in hatchery is conducted at Ifremer/COP/Tahiti. One of the preliminary steps consists in the maturation of the broodstock under laboratory conditions and the results presented here provide information on the nutrition and physiology aspects of the problem. Some tools (respirometer, analytical procedures) provide physiologic measurements (respiration, excretion) taken from animals maintained in the laboratory. Adults were placed in raceways with a flow

through system (0.5 m³/hour) and compared in two situations. Animals received unfiltered water lagoon or the same water plus a regular supply of algae (*Chaetoceros* or T-iso or *Pavlova*) at a concentration of 20,000 cells per ml for a period of 8 weeks at 27 °C.

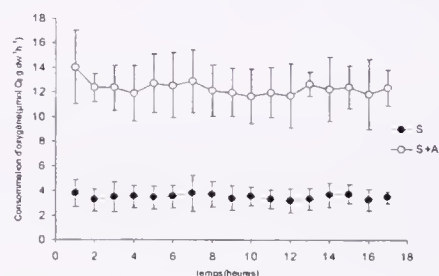


Figure 1. Oxygen consumption recorded on adults placed in a microcosm for 20 hours and reflecting two different nutritional status (seston or seston + algae).

Oyster without additional food respired at a lower level (4 $\mu\text{mol O}_2/\text{jour}/\text{individual}$) than animals with a supplemental algae ration (14 $\mu\text{mol O}_2/\text{jour}/\text{individual}$). Additional measurement of ammonia excretion and a calculation of feed intake helped to produce a scheme of energy partition during the gametogenesis period, moreover it was possible to calculate an O:N ratio that differed between the two conditions of acclimation. Then, energetic substrate changed.

By and large, oysters received a net energy (NE) of 1.5kJ/oyster/day versus 5.0 kJ/oyster/day in a situation with additional food. Nutritional status for animals placed in raceway with a high renewal water and microalgae distributed daily is significantly improved. Animals with supplemental food produced higher indices of gametogenesis (gonadic indices) than the others.

A benefit through the supply of essential nutrients such as DHA, EPA, arachidonic acid, sterols, and phospholipids needed during this phase of gametogenesis is probably part of the explanation of the results from a nutritional point of view and need further analytical work.

PHYSIOLOGICAL ECOLOGY OF THE CULTURED HARD CLAM, *MERCENARIA MERCENARIA*. Elizabeth S. Darrow, Mark W. Luckenbach, and Roger Mann, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA. E-Mail: edarrow@vims.edu

Aquaculture of the hard clam, *Mercenaria mercenaria*, is one of the largest agricultural industries on the Eastern Shore of Virginia. Over the course of development of this industry in the region, marked improvements in clam growth rates were initially realized, as a result of better site selection, improved growing

techniques, selective breeding, or some combination of these factors. In recent years, some decline in clam growth rate has been reported by the industry and there is speculation that the carrying capacity of some grow-out areas has been exceeded. As part of a larger project to evaluate the limits on clam growth rates in this system, we developed an energy budget for these clams that have been selected for fast growth.

We hypothesized that aquacultured clams in Cherrystone Inlet, a tidal creek tributary of the Chesapeake Bay, would have different growth rates than wild clams, owing to changes in either (1) energy acquisition processes (ingestion rates) or (2) energy expenditures (metabolic, excretion, and egestion rates). Ingestion and egestion rates were determined using a flow-through system at *in situ* temperature and salinity, using natural seston from Cherrystone Inlet. After a 3-hour feeding trial, water samples were collected from each chamber's inflow and outflow tubes and analyzed for chlorophyll, cell counts, and total suspended solids. Ingestion rates were calculated by measuring the change in chlorophyll, particles, and particulate organic carbon (POC). Egestion rates were measured by collecting feces from each chamber and determining POC content. Assimilation and growth efficiencies were calculated for each individual. Metabolic rates were determined for the same temperature and salinity range by measuring oxygen depletion due to clam respiration in a gas-impermeable chamber using polarographic oxygen sensors. Simultaneously, excretion rates were calculated from the change in dissolved inorganic nitrogen (DIN) in the static system. All physiologic rates were measured for the full range of cultured clam sizes, from approximately 15 to 50 mm. Clam growth rates were measured on a monthly basis in the field for the same size range.

Results indicate that ingestion rates of cultured clams lie within expected ranges according to previously existing generalized hard clam and bivalve feeding models. This study also implicates a few potential sources of variation in clam ingestion rates, including seston (food) concentration and tidal cycle stage. These factors may be responsible for the observed bimodal distribution of ingestion rates among bivalves known as "high gear" and "low gear".

EVALUATING THE AQUACULTURE POTENTIAL OF ARCTIC SURFLCLAMS (*MACTROMERIS POLYNOMA*) IN THE GULF OF MAINE. Christopher V. Davis and Sandra E. Shumway, Department of Marine Sciences, University of Connecticut, Gton, CT 06340.

The Arctic surf clam *Mactromeris polynoma* (Stimpson 1860), is a cold water circumboreal species distinguished from other surfclams by its purple foot, siphon, and mantle edge that turn brilliant orange-red upon being cooked. Increased demand for wild surfclams to supply the Japanese sushi market prompted a preliminary investigation of the aquaculture potential for cultivation of this species in Gulf of Maine waters. Studies were undertaken to determine the feasibility of rearing this species from biologic, technical, and commercial perspectives.

Hatchery-based studies to determine optimal rearing conditions documented early embryonic development, larval growth, and survival of replicate cohorts cultured under various environmental conditions. Laboratory and field grow-out studies investigated the effects of substrate type and growing site on juvenile growth and survival. A laboratory-based feeding experiment evaluated the uptake and release kinetics of surfclams when fed bloom concentrations of the toxic dinoflagellate (*Alexandrium tamarense*). Finally, a marketing study determined farmgate price, niche markets, and acceptability of fresh, farm-raised surfclams in the North American market sector. Results from these studies and the implications for aquaculture production are discussed.

EFFECT OF A DIETARY CONDITIONING USING LIPID EMULSION ON IMMUNE RESPONSE OF THE PACIFIC OYSTERS *CRASSOSTREA GIGAS*. Maryse Delaporte, Fu-Lin Chu, Chris Langdon, Eric Lund, Vincent Encomio, Georgeta Constantin, Philippe Soudant, and Jean-François Samain, Ifremer Brest Center, BP 70 29280 Plouzané, France. E-mail: Maryse.Delaporte@ifremer.fr

A preliminary study showed that dietary conditioning with three different algae of different fatty acid profiles affected the immune functions of the Pacific oysters *C. gigas* as well as the manila clams *R. philippinarum*. This suggests that a relationship between the essential polyunsaturated fatty acid (PUFA) profile of the diet and immune functions seems to exist in mollusks as in vertebrates. Nevertheless, the effect of individual PUFAs on the immune function cannot be clearly established. So we decided to test the effect of each PUFAs by using artificial diets, and we first tested the effect of 20:5(n-3) PUFA on the immune functions of oysters *C. gigas*.

To do this, oysters were fed either T-*Iso* alone or T-*Iso* supplemented with lipid emulsion during 1 month (mid-February to mid-March 2003). EPAX 4510 TG, an oil marine mixture of triglycerides rich in 20:5(n-3) (45%) and 22:6(n-3) (10%), was retained as the lipid emulsion. Different levels of emulsion were added continuously with the algae to the flow-through seawater: 0%, 1%, 10%, and 50% of the lipid emulsion. Moreover, at the end of the conditioning, 3 weeks of starvation were applied. During the experiment, different immune parameters were followed using a flow cytometer: hemocyte concentration, viability of cells, phagocytosis and the "oxidative burst" activity of hemocytes. At the same time, gills were removed from oysters that hemolymph were sampled for measurement of immune parameters to determine the effects of dietary conditioning on the lipid profile of gill membrane.

Preliminary results showed that the dietary conditioning was efficient: oysters maintained their condition index. However, the highest level of supplementation (50%) did not result in higher condition index than other treatments.

The dietary conditioning affected the immune system of oysters. Oysters fed 0% and 1% emulsion had similar hemocyte con-

centrations, whereas oysters fed 10% and 50% emulsion exhibited significantly higher hemocyte concentrations (Fig. 1). Results also revealed significant differences in phagocytic activities among treatments.

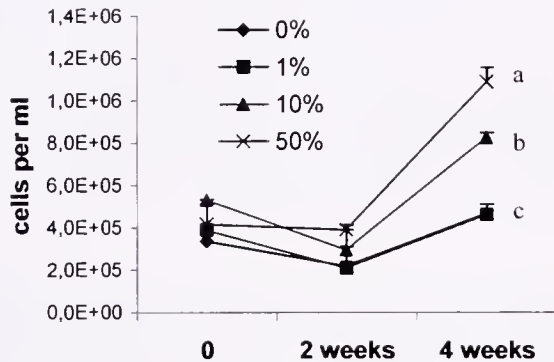


Figure 1. Haemocyte concentrations of oysters during the dietary experiment. Small letter indicate significant difference between dietary treatments (ANOVA, $p < 0.05$).

AUSTRALIAN SCALLOP SEABED CULTURE: FROM CONCEPT TO COMMERCIALIZATION. P. F. Duncan, S. Wang, E. O'Brien, W. Knibb, B. M. Degnan, and T. Wittingham, Faculty of Science, University of the Sunshine Coast, Maroochydore DC, Queensland 4558, Australia. E-mail: pduncan@usc.edu.au

The Amusium balloti fishery is one of Queensland's most valuable, but has varied in value between A\$15–24m/annum in the last decade. Although variability is common in scallop fisheries, the recent Queensland trend has been declining CPUE. Also, pressure to reduce environmental trawling impacts has changed fisheries management policy and resulted in a >30% fleet reduction. To maintain the economic and social benefits associated with the fishery, alternative production and management options are being considered with particular interest in aquaculture and ranching.

Wild spat collection and larviculture has been limited until recently, restricting aquaculture development, but advances in Queensland and Western Australia have enabled seabed culture projects in both states. The Queensland project is reviewed.

Since 1999, spawning, larval development, culture environment (eg, temperature and feed), larval settlement and attachment, and post-settlement behavior have been investigated. Spawning induction is via heat shock, early larval growth is highest at 20 °C, with survival highest at 18 °C. Larval survival and growth are better with multi-species algal diets, although Tahitian *Isochrysis galbana* (T-iso) alone gave good results. Artificial chemical cues increased larval settlement and metamorphosis, but substrate type had no effect. Biofilm exposure increased metamorphosis, but also mortality rates. Downwelling systems improved survival com-

pared with traditional settlement tanks and continuous aeration is essential. The larval cycle is about 25 days.

Current research is addressing the population genetics and taxonomic status of Western Australian and Queensland wild saucer scallop populations. Understanding the existing population structure is an essential component of a responsible marine release program. Previous allozyme work has indicated a probable taxonomic differentiation between west and east coast populations. Cytochrome oxidase and mtDNA markers will be applied to this question, which may have important implications for larval production, release and management strategies in the two programs.

A key component in project development has been the collaboration between industry and researchers. Queensland Sea Scallop Ltd. was formed in 2002 and has recently obtained the first seabed licenses for aquaculture in Queensland.

PROGRESS IN THE DEVELOPMENT OF EFFECTIVE PROBIOTIC BACTERIA FOR BIVALVE SHELLFISH HATCHERIES AND NURSERIES. Ralph Elston, Karen Humphrey, Arthur Gee, Daniel Cheney, and Jonathan Davis, AquaTechnics, PO Box 687, Carlsborg, WA 98324, USA. E-mail: aquatech@olympen.com

In small-scale laboratory systems we have tested the efficacy of multiple probiotic bacterial candidates to protect Pacific oyster (*Crassostrea gigas*) larvae and juveniles against challenge by bacterial pathogens and to increase survival of unchallenged hatchery-produced larval oysters. Studies focused on three promising bacterial strains isolated in the region of the shellfish production facilities. In well plates with 12 replicates, probiotic candidate P02-45 added at a concentration of 10^5 cfu per mL resulted $84.9\% \pm 4.8\%$ (1 SD) survival in larvae challenged with 10^4 cfu of a moderately pathogenic *Vibrio*, compared to $44.6\% \pm 4.2\%$ survival in challenged larvae without probiotic added. We repeated this experiment on additional spawn groups of larvae with similar results. We also found that addition of the probiotic candidate P02-45 added to unchallenged hatchery production batches of larvae could result in a survival improvement as great as from 21.0% without probiotic to 76.5% with probiotic when tested over 48 hours.

Tests are underway to scale up the test volumes and test the efficacy of killed bacteria. We also evaluated the compatibility of probiotic candidates P02-45 and P02-1 with seven species of algal food. We found that these probiotic candidates were compatible with some algal foods and even resulted in an increased density of food cells. Two algal species compatible with the probiotic candidates were Tahitian *Isochrysis* and *Rhodomonas* sp. (Fig. 1). Examination of the bacterial concentrations in these algal cultures showed that they reached a range of 10^5 – 10^6 cfu/mL, an effective concentration for providing probiotic protection. Addition of algal

cultures containing probiotic bacteria provided protection for larvae challenged with pathogenic *Vibrio* sp.

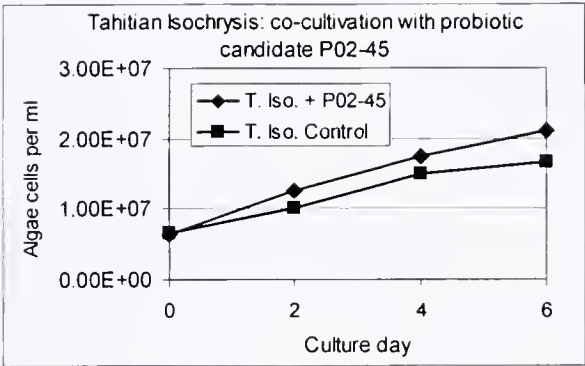


Figure 1.

We tested the highest concentration of probiotic that could be tolerated by oyster larvae and found it to be approximately 10⁷ cfu/mL. Above this concentration, the larvae appeared to die from anoxia because of the high bacterial concentration. We also tested the primary probiotic candidates for pathogenicity to larval manila clams (*Venerupis philippinarum*) and larval geoduck clams (*Panope abrupta*) and found them to be non-pathogenic.

Research supported by grant project number 2002-00362 from the US Department of Agriculture, Cooperative State Research, Education, and Extension Service, Small Business Innovation Research program.

CHARACTERIZATION OF HEAT SHOCK PROTEIN EXPRESSION AND INDUCED THERMOTOLERANCE IN *P. marinus* PARASITIZED EASTERN OYSTERS: LAB AND FIELD STUDIES. Vincent G. Encomio*; Fu-Lin E. Chu, Virginia Institute of Marine Science College of William and Mary Gloucester Point, VA 23062 vge@vims.edu

Eastern oyster mortalities caused by *Perkinsus marinus* (Dermo) are likely exacerbated by thermal stress. Conversely, enhancing thermotolerance in oysters may improve tolerance to thermal stress and disease. Sublethal heat shock has been found to increase tolerance to subsequent lethal heat stress (induced thermotolerance) in oysters. Increased heat shock protein (hsp) expression has been associated with induced thermotolerance. Heat shock proteins are also believed to play a role in protecting organisms from pathogenic stress. The objectives of our research are to characterize the heat shock response in the eastern oyster and deter-

mine its role in mediating stresses due to high temperature and disease.

To examine the interaction between thermal stress and parasitism, oysters were challenged with *Perkinsus marinus* and maintained until infection levels reached a body burden of 10⁴ cells/g wet weight. Oysters were then heat shocked for 1 h at 40°C. Gill tissues were sampled at 0, 2, and 7 days post heat shock and analyzed for total hsp70 levels. At day 7, oysters were subjected to a lethal temperature of 45°C for one hour and survival was monitored for one week after lethal heat stress. Survival in heat shocked oysters was significantly higher than non-heat shocked oysters (*P* = 0.013). Infection status did not significantly affect survival. Despite an apparent induced thermotolerance, gill hsp70 levels decreased significantly over time (*P* = 0.003), counter to expected results. Further analyses will be conducted to determine if variability in isoform expression accounts for these changes. Seasonal hsp70 levels are also being compared in field deployed disease resistant and susceptible oyster stocks. Preliminary results show

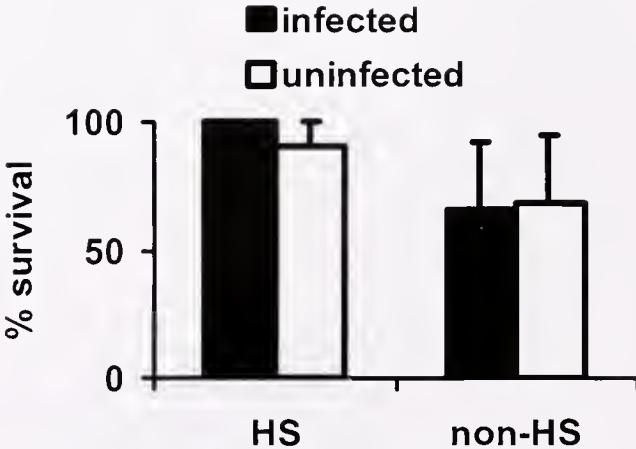


Figure 1. Percent survival of oysters after lethal heat stress. HS = heat shock; non-HS = non-heat shocked.

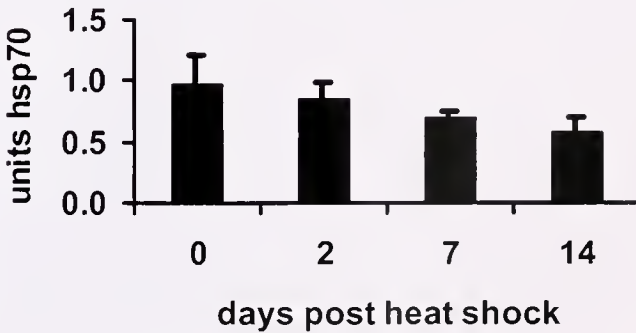


Figure 2. Total gill hsp70 levels after heat shock.

that there is little variation in total hsp70 among stocks. Seasonal and induced expression of hsp70 will be compared among these stocks to determine the role of hsps in stress and disease tolerance of disease resistant oysters.

PRIORITIZING REGIONAL RESEARCH INITIATIVES FOR THE CONSERVATION OF ARKANSAS' FRESHWATER MUSSELS. Jerry L. Farris, John L. Harris, Bill Posey, and Alan Christian, Arkansas State University, Department of Environmental Sciences, Box 847, State University, AR 72467, USA. E-mail: jlfarris@astate.edu

Since summarizing Arkansas' freshwater mussel surveys over the past 6 years, questions have been raised concerning how best to utilize the data in developing successful management strategies for this declining group of aquatic invertebrates. Projects targeting habitat requirements, life history assessments, and propagation support have drawn upon our mussel database. This database was developed for GIS application to offer insight for best management practices and decision-making when resource sustainability and environmental protection are prioritized. It seems that priorities continue to demand an efficient level of collaboration among researchers and managers to insure meaningful progress towards answering the diverse range of questions concerning conservation planning. A review of the synergism in these conservation objectives and outcomes offers insight into the importance of prioritization of resources.

Initial large-scale surveys to delimit aerial extent of commercial mussel beds have led to improved coordination on river navigation maintenance activities such that the United States Army Corps of Engineers preapproval for dredging and disposal has resulted in significant protection of mussel resources. This in turn has led to an expanded effort for educating and equipping managers for identifying areas to support restoration and recovery of species such as *Potamilus capax*. Awareness of this mussel's distributions has even accounted for field validations of prescribed buffer zones for eradication of the boll weevil in agricultural production.

Developmental objectives related to efficiently handling, transporting and holding captive species have elucidated the importance of suitable refugia and hatcheries in supporting long-term observational and manipulative studies. The success of those efforts has now furnished important life history information and propagation of four different listed species. Attention is currently shifting to species of *Lampsilis*, *Pleurobema*, *Fusconaia*, *Cyprogenia*, and *Quadrula* to resolve important relationships utilizing phylogenetic assessments. All of these efforts have benefited from the coordination and dissemination of information by the Arkansas Fresh-

water Mussel Initiative. Such workgroups are key to making informed decisions in the face of limited budgets and critical time frames.

INORGANIC ELEMENTS AND DISTRIBUTION OF EASTERN OYSTERS. William S. Fisher, US Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, 1 Sabine Island Drive, Gulf Breeze, FL 32561, USA

For over a century we have marveled at the high concentrations of inorganic elements, particularly zinc and copper, in oysters. Many have suggested that accumulations were inadvertent because the concentrations were too high to serve any known physiologic function. Recent evidence, however, has led to a hypothesis that eastern oysters *Crassostrea virginica* depend on substantial quantities of inorganic elements to serve in antimicrobial defense and shell deposition. Integral to this hypothesis is the understanding that ameboid blood cells (amebocytes) and adult shells can actively concentrate inorganic elements thousands of times over ambient.

Amebocytes in the mucus and gut of oysters seem to concentrate zinc, copper, and possibly tin, iron, manganese, and other elements from food and the water column. Discounting shell concentrations, zinc and copper are retained almost exclusively (?90%) in the amebocytes. Sequestration in membrane-bound amebocyte granules prevents toxicity and may provide storage until the elements are released in a defensive capacity. Extracellular release of copper and zinc (via degranulation) has been demonstrated during repair of wounds; they may serve as antimicrobial agents and initiate extracellular clotting. High metal concentrations released within a small area would provide a potent cytotoxic action against either potential pathogens or food organisms. An intracellular release of metals may occur similarly during phagocytosis when granules (lysosomes) fuse with particle-laden phagosomes. Adult oyster shells also contain high concentrations of the same inorganic elements that are retained in the amebocytes. High shell concentrations support a hypothesis that release of the elements from amebocytes initiates precipitation within the matrix of the organic conchiolin, leading to shell calcification. Amebocyte involvement in shell repair and pearl formation has been demonstrated in other molluscan species.

If eastern oysters are, in fact, dependent on high concentrations of inorganic elements, then they must inhabit areas that provide a source. Oysters are marine animals, but the elements are derived from terrestrial sources. Hence, oysters must live in close proximity to land or to freshwater flows that transport the elements. Oyster distributions match this supposition; they exist close to land and venture into deeper waters only within zones of freshwater influence.

WAS/NSA/AFS TRIENNIAL MEETING HAWAII 2004: EAST MEETS WEST. Gel Flimlin, Rutgers Cooperative Extension.

For the first time, members of the well-established Pacific Coast Shellfish Growers Association and the newly formed East Coast Shellfish Growers Association meet to discuss issues of mutual interest. The program will consist of discussions about Federal permitting of shellfish culture, foreign interest in US shellfish markets, organic labeling of cultured shellfish, assessing successful agricultural or aquaculture organizations, tempering harvested clams, interstate or interregional shipping of shellfish seed, update on post harvest treatment of shellfish, using a marketing board to improve profits, and environmental impacts of shellfish aquaculture. The two groups also address topics on which they can collaborate, and where they are focusing their energies presently.

ECONOMIC FEASIBILITY OF SMALL-SCALE, COMMERCIAL CULTURE OF BLACK PEARLS IN RURAL COMMUNITIES IN THE CENTRAL PACIFIC. Quentin S. W. Fong, Fishery Industrial Technology Center/Marine Advisory Program, University of Alaska Fairbanks, 118 Trident Way, Kodiak, AK 99615, USA; Simon C. Ellis, Mid-Pacific Marine Consultants, P. O. Box 2031, Kolonia, Pohnpei FM 96941, Federated States of Micronesia; and Maria Haws, University of Hawaii at Hilo, 200 W. Kawili St., Hilo HI 96720.

Traditional revenue sources for island nations in the Central Pacific such as copra have diminished significantly. Further, many of the high valued natural resources such as wild groupers for the live fish markets and sharks for their fins for the Asian markets, particularly Hong Kong, are being exploited and over-harvested by foreign fleets, using foreign labor with little economic benefits to the local population. With the dwindling of such natural resources, aquaculture is being considered and implemented from high-school education level to industry level to supplement the economic viability of outer island communities throughout Central Pacific.

This work provides an analysis of the economic feasibility of one of many small-scale aquaculture operations being considered, black pearl oyster farms, as one type of supplemental economic activity for outer island communities in the Republic of Marshall Islands. Specifically, projections of financial performance of a small-scale 25,000 seeded pearl oyster farm using the Tahitian long-line method are being conducted. Estimates of initial capital investment and annual operating costs are being formulated; an annual cash flow and enterprise budget are being developed. Preliminary results show that initial capital investment is approximately \$203,030. Annual operating costs is about \$221,212.

Net returns over a 20-year farm horizon average is \$128, 223 per year, based on the most conservative price data from published

sources. Sensitivity analysis on profit due to the variability of market price, survival, and cost of seed and other inputs will be conducted and results presented.

EFFICACY AND DEPLETION DYNAMICS OF OXYTETRACYCLINE USED IN THE TREATMENT OF WITHERING SYNDROME IN CALIFORNIA ABALONE *HALIOTIS* SPP. Abbreviated title: EFFICACY AND DEPLETION OF OXYTETRACYCLINE IN ABALONE WITH WS. Carolyn S. Friedman, Thea T. Robbins, George Trevelyan, Ray Fields, Eric Rosenblum, James D. Moore and Ronald S. Tjeerdema, School of Aquatic and Fishery Sciences, University of Washington, 1122 NE Boat Street, Seattle, WA 98195, USA. E-mail: carolynf@u.washington.edu

Withering Syndrome (WS) is a lethal bacterial disease of wild and cultured abalone, *Haliotis* spp., along the west coast of North America. Therapeutic treatment of WS was assessed using oxytetracycline (OTC) by injection and *per os* administration. Survival of OTC-injected abalone was significantly higher than that of sham-treated animals ($P < 0.001$ and $P < 0.05$). Microscopic examination revealed that all the animals that died had advanced WS and that tissue damage was beyond the ability of the animals to repair in the treated group. Feeding rates of treated abalone were also significantly higher than for sham treated control animals ($P < 0.001$ for black abalone and $P < 0.0001$ for red abalone). Oral administration for 14 consecutive days also effectively controlled WS in pilot and production scale applications. The treatment caused significant and persistent long-term reductions in the intensity of RLP infection and in the degree of morphological changes in the digestive gland ($P < 0.001$, $P < 0.05$, respectively) in treated individuals. On a pilot scale, using a 14-day treatment, mortality dropped from 36% in the control groups to 5% in the treated populations ($P < 0.001$). On a commercial scale, a dry floating feed containing 2.6% active OTC was just as effective. Withering syndrome caused high (41%) losses in control production tanks, but treated tanks experienced only 9% mortality ($P < 0.001$). Treated tanks produced 56% more market-sized abalone than control tanks ($P < 0.001$) resulting in 70% greater biomass of market-sized abalone relative to control tanks ($P < 0.01$). Concentrations of OTC in individual abalone foot muscle were variable and peaked at over 40 ppm. Residues declined to < 2 ppm, the federal tolerance level, between 15 and 22 d post treatment. The digestive gland however, appears to concentrate OTC and residence times have exceeded 3 mo based on a preliminary examination with levels as high as ~20–180 ppm. Based on these data, we designed a study to optimize the efficacy of the OTC treatment (for 10, 20, or 30 d) and characterized the depletion dynamics of treated red abalone up to 122 d post treatment.

SEASONAL AND TEMPORAL VARIABILITY IN TISSUE BIOCHEMISTRY OF SEVERAL FRESHWATER MUSSEL SPECIES. Catherine M. Gatenby, Julie Boyles, Danielle Kreeger, Deborah Raksany, and Richard Neves, United States Fish and Wildlife Service, White Sulphur Springs National Fish Hatchery, WSS, WV 24986, USA. E-mail: Catherine_Gatenby@fws.gov

Freshwater mussel populations are highly imperiled worldwide. In the United States, approximately 70% of native species are in serious decline because of chronic environmental degradation, toxic spills, and the invasion of the exotic zebra mussel (*Dreissena polymorpha*). All 70 endangered mussel species recovery plans call for propagation and reintroduction of populations to achieve recovery. Additionally, propagation technology is viewed as a recovery mitigation tool for mussels killed by toxic spills or other anthropogenic impacts. To support successful propagation, baseline information on the nutritional demands of wild freshwater mussels is needed in order to develop suitable diets and feeding regimes for maintaining mussels in captivity.

Our goal is to quantify variability in physiologic status of several species of mussels from varying drainages and from different subfamilies. Proximate tissue biochemistry (protein, lipid, carbohydrate, and ash) was monitored in healthy populations of mussels collected from rivers in the Atlantic and Ohio River drainages over a 3-year period. Biochemistry varied seasonally and temporally across the years of study. Biochemistry also differed between the subfamilies of mussels. The mean carbohydrate level (%DTW) of each species was similar; whereas, the relative protein and lipid levels differed between species. These results reflect the reproductive and seasonal conditioning behavior of these animals, and their responsiveness to environmental changes. Until further information becomes available on absolute nutritional requirements, we recommend the following dietary composition for captive freshwater mussel diets: ~5%–10% carbohydrate, ~20%–40% protein, ~30% lipid, and ash ~20%.

VARIATION IN GROWTH AND REPRODUCTION OF BAY SCALLOPS FROM SIX SUBPOPULATIONS FROM THE NORTHEAST GULF OF MEXICO. Stephen P. Geiger, Janessa Cobb, and William S. Arnold, Florida Marine Research Institute, 100 8th Ave. SE, St. Petersburg, FL, 33701, USA. E-mail: steve.geiger@fwc.state.fl.us

Bay scallops (*Argopecten irradians*) can be found over a large geographic range: from Nova Scotia to Columbia. At the northern end of its range, reproduction occurs during the warm summer months. In the mid-Atlantic states, reproduction occurs when waters warm during the spring. In Florida, reproduction is conventionally believed to occur as water temperatures decline in the fall. Growth will often be limited to one body compartment: either somatic or reproductive. Often, somatic tissue weight will decline during the peak of reproductive growth. We examined variation in both tissue and somatic growth over a small geographic range: six

subpopulations of bay scallop along Florida's west coast. Beginning in April of 2002, we collected sets of up to 30 scallops in each of six subpopulations: St. Joseph Bay, Lanark, St. Mark's River, Steinhatchee, Homosassa, and Anclote. The collections were conducted at least once per month, but were conducted more often when possible, and continued through September. We determined shell height, wet weight and dry weight of adductor muscle, gonad, and all other tissue (hereafter termed viscera) and the volume of each adductor muscle.

One population, Lanark, was generally larger in all morphologic measures except gonad weight. Otherwise, the southernmost population, Anclote, began the season largest. This fits a general pattern observed in annual measurements of shell height during our spring survey. All populations had an increased gonadosomatic index in September. However, the gonad never exceeded 10% of the total tissue weight in the Anclote population, whereas gonads exceeded 20% of the total tissue weight in St. Mark's and St. Joseph Bay. Growth as measured by changes in mean shell height of the population was also higher in the three populations, which started smallest (St. Mark's, Steinhatchee and St. Joseph Bay; 4–6 mm/month), than the others (2.4–2.6 mm/month Homosassa, Anclote, and Lanark).

A possible explanation for our findings is that growth in the populations of bay scallops found in Florida's panhandle region more closely resemble those found in true temperate climates. They exhibit short periods of rapid growth and distinct spawning events. Those populations along peninsular Florida maintain slower growth throughout the year, but have more protracted growth and spawning seasons. Evidence from studies of juvenile recruitment is presented to support this theory.

ABUNDANCE OF HARD CLAM LARVAE IN THE INDIAN RIVER LAGOON. Stephen P. Geiger and William S. Arnold, Florida Fish & Wildlife Conservation Commission, Marine Research Institute, 100 8th Ave. SE, St. Petersburg, FL 33701, USA; and Marc Frischer, Skidaway Institute of Oceanography, 10 Ocean Science Circle, Savannah, GA 31411, USA.

The Indian River Lagoon complex, located on Florida's east coast, is a 220-km long system of three connected water bodies (the Mosquito Lagoon, the Banana River, and the Indian River) with both limited freshwater input and limited tidal exchange with the Atlantic Ocean. A naturally low circulation is further restricted by a series of man-made causeways connecting the mainland with the barrier islands that create the lagoons. Hard clams have been harvested from the lagoon since pre-historic times. Two periods of peak harvest dominated the commercial fishery, one in the mid 1980s and a second in the early 1990s. During each period, the value of the annual harvest peaked near \$8,000,000 (~0.5 million bushels). Current annual harvest is around 50,000 pounds per year. One characteristic of the fishery is that these rare sets that produce commercially harvestable densities of clams occur in highly local-

ized areas: usually a single water body within the complex. We have hypothesized that because of the limited circulation, larval supply is severely limited, reducing the opportunity for good sets of new recruits, especially in areas where adult densities are low. Unfortunately, identification of bivalve larvae, even to the genus level, or monitoring recruitment of juveniles is tedious and difficult.

To test the hypothesis of limited larval supply, we have been measuring the abundance of hard clam larvae through the use of a genetic probe. The 18S rRNA targeted oligonucleotide probe is specific to *Mercenaria* spp. (the Indian River has *M. mercenaria*, *M. campechiensis*, and hybrids between the two species). Use of this probe allows semi-quantitative estimates of larvae in unsorted plankton samples. The procedure entails extracting the total nucleic acid pool from the sample, binding it to a charged membrane, and visualizing the species-specific probed RNA on film after labeling with radioisotopes (in clams) or chemiluminescent compounds (for example in scallops). In order to test the efficacy we compared the technique to direct counts of bivalve larvae from sub-samples and also to a series of standards from cultured clam larvae.

Results from six sampling efforts in the Indian River Lagoon during the years 1998 to 2003 show that larval hard clam abundance in the complex is generally less than 20 larvae per liter but can vary spatially and temporally. Within a single sample period, the range of larval density from all stations is typically less than one order of magnitude. Variation between sample periods show that larval density can range from times when no larvae are present to times when larvae were detected at more than 90% of the stations sampled. Total bivalve larvae reach levels of hundreds per liter. The genetic probe method suggests that hard clam larvae usually represent <10% of the bivalve larvae present.

This technique offers a means by which researchers and managers can monitor larval abundances in natural systems and can explore the questions of whether larval supply is limiting or whether other life stages are critical in maintaining populations.

ENVIRONMENTAL CONDITIONS AND EXPERIMENTAL INFECTION WITH *P. MARINUS* MODULATE CELLULAR DEFENSE MECHANISMS IN THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*. Michael Goedken and Sylvain De Guise, Department of Pathobiology and Veterinary Science, University of Connecticut, 61 North Eagleville Road, U-89, Storrs, CT 06269, USA. E-mail: michael.goedken@uconn.edu

The fast growing oyster aquaculture industry is greatly hindered by *Perkinsus marinus* and *Haplosporidium nelsoni*, which can kill up to 80% of production. The relationship between parasites, oyster defense mechanisms and their environment is unclear.

Phagocytosis (using fluorescent beads) and apoptosis (using Annexin-V FITC) of Eastern Oyster (*Crassostrea virginica*) hemocyte subpopulations were quantified at the single cell level utilizing flow cytometry. The influence of salinity (428 mOsm or 1.006 ‰ vs. 1006 mOsm or 1.025 ‰) and temperature (11 °C vs. 25 °C) were evaluated experimentally. Forward and side scatter distinguished two populations of hemocytes (granulocytes and hyalinocytes) with previously demonstrated unique functional characteristics. Controlled environment and infection studies revealed significant immune function alterations. Phagocytosis in granulocytes, but not in hyalinocytes, was higher at low salinity (428 mOsm or salinity of approximately 1.009) than at high salinity (1006 mOsm or salinity of approximately 1.025) when the water temperature was 25 °C. Phagocytosis was not affected by salinity when the water temperature was 11 °C. Temperature had no effect on hemocyte phagocytosis at either salinity. Apoptosis in both granulocytes and hyalinocytes was higher at 11 °C than at 25 °C when the water salinity was low (428 mOsm). Temperature had no effect on hemocyte apoptosis when salinity was high (1006 mOsm). Salinity had no effect on hemocyte apoptosis at either water temperature. Apoptosis in both granulocytes and hyalinocytes was higher upon *in vitro* infection with *P. marinus* compared to uninfected control cells. We demonstrated experimentally that water salinity, water temperature, and experimental infection with *P. marinus* modulated oyster defense mechanisms. A better understanding of how these variables affect oysters' defense mechanisms may lead to management strategies that will result in reduced disease morbidity and mortality for oyster producers.

WORLD ABALONE FISHERIES AND AQUACULTURE UPDATE: SUPPLY AND MARKET DYNAMICS. H. Roy Gordon, Fishtech Inc., Box 6886, San Rafael, CA 94903, USA. E-mail: rgordon@fishtech.com and Peter A. Cook, Center of Excellence in National Resource Management, University of Western Australia, Albany, 6330, Australia.

Continued increases in cultured production, combined with an unfortunate proliferation in the illegal wild catch have resulted in a world abalone supply for the year 2002/2003 exceeding the historical abalone abundance of the mid 1970s. Sparked by the rising middle class in China, a global shift in abalone availability and distribution has occurred. These significant shifts demand inventive thinking on the supply side if prices are to remain at strong levels. Examples are given of approaches using new creativity in the world of abalone marketing, distribution, and processing.

FAO country by country abalone reporting for both wild caught and cultured abalone totals change significantly when further dissected and standardized. FAO practice of reporting totals for abalone shucked or not and the continuing practice of some countries still reporting their production by large groups of species, distorts

historical and current totals. The information that we present on worldwide legal and illegal wild catch and on cultured production has been adjusted to account for these anomalies.

AN EXPERIMENTAL TEST OF LARVAL SOURCE AND CULTCH TYPE ON CONSTRUCTED OYSTER (*CRASSOSTREA VIRGINICA*) REEF PERFORMANCE Jennifer Greene* and Ray Grizzle, Department of Zoology University of New Hampshire Durham, NH 03824 jenn.greene@unh.edu

The roles that the eastern oyster (*Crassostrea virginica*) plays economically and ecologically are of substantial importance in the Great Bay estuary (NH) where there has been dramatic declines in oyster abundances since the mid 1990s when the first MSX epizootic occurred. The study of oyster reef restoration techniques is a high priority goal in the New Hampshire Estuaries Project Management Plan. In accordance with this, the current research project was developed to test the following hypotheses: (1) Reefs constructed using disease-resistant spat perform better than reefs formed by native spat; and (2) Cultch material affects remote setting success and early reef performance. This research project also addresses the goals set for managing oyster populations both locally and Bay-wide but also with implications for other Northern estuaries with oyster populations. These hypotheses will be tested by constructing 12 randomly positioned mini-reefs adjacent to an existing oyster reef using a $2 \times 2 \times 3$ factorial design. Each mini-reef ($\sim 2 \times 3$ m) will consist of a combination of two experimental variables: larval source (Maine native and NY disease-resistant) and cultch type (concrete/granite and oyster shells). Each spat/cultch combination will be replicated three times. To provide information on spat set on live oysters, the existing reef at Adams Point will be sampled at the same intervals as the experimental reefs. The larvae were remotely set in July 2003 and reef construction is planned for October 2003.

Each group of larvae was set at Jackson Estuarine Laboratory individually (5,000,000 per set) by cultch type in two 3,000-gallon fiberglass tanks. Initial larval settlement densities for the native Maine broodstock on the concrete/granite cultch were 9.1% while the oyster shell had a higher settlement rate of 16.5%. Initial larval settlement for the NY disease-resistant larvae was 4.8% on the concrete/granite cultch and 1.6% on the oyster shells. After settlement, cultch bags were transferred to rafts anchored in an intertidal nursery area in a sheltered cove adjacent to the Laboratory. Growth and mortality will be measured on a weekly basis while in the nursery area. After transfer to reef area in early Fall, growth and mortality will be measured bi-monthly.

The overall goal of this research is to provide information useful for the design of an estuary-wide oyster reef restoration program. The State of New Hampshire has set a goal of restoring 20 acres of oyster bottom by 2010.

STRATEGIES FOR MAPPING DISEASE-RESISTANCE GENES IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA* GMELIN. Ximing Guo, Ziniu Yu, Yongping Wang, and Susan Ford, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA. E-mail: xguo@hsrl.rutgers.edu

The eastern oyster, *Crassostrea virginica* Gmelin, faces two major diseases along much of the Atlantic coast: MSX (caused by *Haplosporidium nelsoni*) and Dermo (caused by *Perkinsus marinus*). Resistance to the two diseases has a genetic component and can be enhanced by selective breeding. The mapping of disease-resistance genes may improve selection efficiency through marker-assisted selection. Mapping disease-resistance genes in oysters faces three challenges: (1) the lack of several genetic markers; (2) the lack of well-defined inbred lines and the difficulty to differentiate susceptible from resistant phenotypes; and (3) inability to harvest their tissues. We have been testing the following strategies for mapping in the eastern oyster. For markers, we relied on amplified fragment length polymorphisms (AFLPs) because they are relatively easy to develop and use. We developed over 500 polymorphic AFLP markers and built a moderately dense linkage map. We constructed 15 reference families using Rutgers disease-resistant strains and oysters from Delaware Bay, Long Island Sound, and Gulf of Mexico to maximize the segregation of markers and disease-resistance genes. Three approaches are being used to identify and map disease-resistant genes. First, we are studying and comparing allele frequencies in selected and wild populations to identify markers that show consistent frequency differences. Secondly, we are screening for allele frequency shifts before and after disease-inflicted mortalities in families and populations. Finally, we are looking for concerted linkage of suspected markers on the genetic map. The use of large number of AFLP markers, multiple reference families, and several mapping approaches may lead to the identification and mapping of some disease-resistant loci in the eastern oyster.

PHYSICAL AND LINKAGE MAPPING IN THE EASTERN OYSTER *CRASSOSTREA VIRGINICA* GMELIN. Ximing Guo, Yongping Wang, and Ziniu Yu, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA. E-mail: xguo@hsrl.rutgers.edu

The eastern oyster, *Crassostrea virginica* Gmelin, supports major fishery and aquaculture industries in the United States. It is also a popular model species for molluscan research. The biology and ecology of the eastern oyster are relatively well understood, but our knowledge about its genome is rather limited. In an attempt to develop a basic genomic map for the eastern oyster, work has been conducted to develop tools for physical and linkage mapping. For

physical mapping, we have been using fluorescence in situ hybridization (FISH) for the cytogenetic characterization and mapping of oyster chromosomes. Many repetitive DNA sequences and genes have been assigned to oyster chromosomes by FISH, and some revealed interesting features of the oyster genome. The chromosomal location of rRNA genes, on the long arms of Chromosome 10 (10q) in all Pacific species and on the short arms of Chromosome 2 (2p) in all Atlantic species studied so far, provides an interesting divide between the Pacific and Atlantic species. Nine unique sequence probes (P1 clones) have been mapped to eight of the 10 oyster chromosomes, and more are being mapped. For linkage mapping, we focused on the development and use of amplified fragment length polymorphism (AFLP) markers. Over 500 polymorphic AFLP markers were developed, and a moderately dense linkage map was built. This preliminary map is being used for the mapping of disease-resistance genes in the eastern oyster. Microsatellite and Type I markers are being added to the linkage map. Segregation markers are being developed for the cytogenetically mapped P1 clones. The goal is to integrate the cytogenetic and linkage maps by assigning the same DNA sequences to specific chromosomes and linkage groups.

EVALUATION OF PHA ON DIATOM COLONIZATION AND GRAZING ABILITY OF RED ABALONE *HALIOTIS RUFESCENS* POSTLARVAE. María T. Gutiérrez-Wing, Carmen G. Paniagua-Chávez, and Ronald F. Malone, Institute for Ecological Infrastructure Engineering, Civil and Environmental Engineering Department, Louisiana State University, 100 CEBA Lane, Baton Rouge, LA 70803, USA.

Biodegradable plastics have been used in areas such as medicine and engineering. The use of these plastics in aquaculture could help greatly to resolve environmental problems or enhance the culture of different species. Polyhydroxyalkanoates (PHA) (Fig. 1) is a biodegradable biopolymer produced by bacterial fermentation of sugar.

The objectives of this work are to (1) evaluate the performance of PHA plates as a substrate for the colonization of diatoms used to feed red abalone post larvae and (2) determine the grazing ability of red abalone post larvae on diatoms attached to the PHA plates. For the first experiment, pieces of PHA (5-cm diameter) were placed separately in 500-mL flasks containing the diatom *Navicula inserta* or seawater. Growth and colonization of diatoms were registered every other day for 20 days. For the second experiment, red abalone post larvae were placed in 20-L buckets containing plates previously colonized with *N. inserta*. As control, plastic plates used commonly to grow post larvae in the hatcheries were used. Initial and final post larvae size and weight were registered to determine growth and body gain.



Figure 1. Scanning electron micrographs of PHA's.

QUALITY SURVEY FOR ALASKAN SHELLFISH IN THE UNITED STATES: IMPLICATIONS FOR MARKETING COOPERATIVE FORMATION. Erin Harrington and Quentin S. W. Fong, Fishery Industrial Technology Center/Marine Advisory Program, University of Alaska Fairbanks, 118 Trident Way, Kodiak, AK 99615, USA.

The volume of aquaculture shellfish produced in Alaska has increased more than 400% since the early 1990s. Increasing saturation of Alaskan shellfish markets has led the industry to look to markets outside the state. The intent of research is to determine shellfish buyers' relative preference various product characteristics of shellfish. Mail surveys and focus groups will be used to determine buyers' and market expectations for Alaskan cultured shellfish and to assess variables such as seasonality of consumption, destination of product, and preferred species and product forms. A conjoint analysis of buyers' preference is being conducted to deconstruct a buyer's overall evaluation of a product into its component attributes such as size, product form, price, and lipid content. Results will be paired with comparative analysis of attributes of shellfish produced in different regions of the state. Study results are intended to assist shellfish producers in gauging the feasibility of regional or statewide marketing cooperatives. Preliminary results will be presented.

A STAGE-BASED POPULATION MODEL FOR BAY SCALLOPS *ARGOPECTEN IRRADIANS* AND IMPLICATIONS FOR POPULATION-LEVEL EFFECTS OF HABITAT ALTERATION. Elizabeth K. Hinchey*, Mamita M. Chintala, and Timothy R. Gleason, U.S. Environmental Protection Agency, Atlantic Ecology Division 27 Tarzwell Dr. Narragansett, RI 02882 hinchey.elizabeth@epa.gov

Bay scallops (*Argopecten irradians*) inhabit shallow subtidal habitats along the Atlantic coast of the United States and require settlement substrates, such as submerged aquatic vegetation (SAV), for their early juvenile stages. The short lifespan of bay scallops (1-2 y) coupled with a dependency on an essential habitat (SAV) renders this species particularly vulnerable to coastal habitat alteration. We are investigating the effects of habitat alteration on bay scallop populations using a stage-based matrix population model, in which the life history of *A. irradians* is divided into five life stages (Fig. 1). By quantitatively representing the life history strategy of the bay scallop and directly incorporating stressor effects and stressor-response relationships for specific life stages

into model projections, the matrix model provides a framework for evaluating the risk that habitat alteration (in the form of decline in SAV habitat quality and quantity) poses to bay scallop populations. Model output relating the response of scallop population growth rate to environmental stressors will be presented, along with simulation results of the effects of habitat alteration on scallop populations. Through elasticity analysis, the model can also be used to evaluate management strategies for population enhancement and identify data gaps most critical to understanding the population dynamics of this economically valuable and dwindling species.

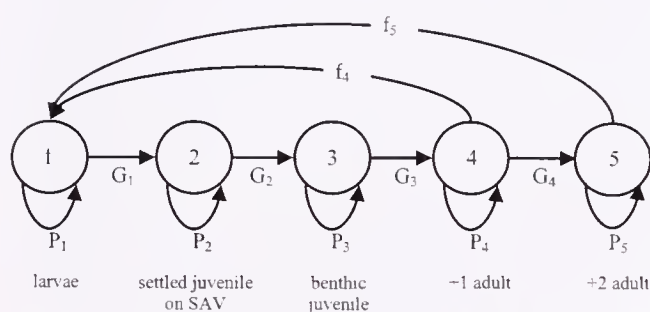


Figure 1. Life cycle representation of the stage-based population model for bay scallop (*Argopecten irradians*) with a weekly time step. G_i represents the probability of an individual surviving to the next stage class during a time step; P_i represents the probability of an individual surviving and remaining in that stage class during a time step; f_i represents the reproductive output of an individual in that stage class during a time step.

DIFFERENTIALLY EXPRESSED GENES IN RELATION TO SUMMER MORTALITY OF THE OYSTER *CRASSOSTREA GIGAS* REVEALED BY SUPPRESSION SUBTRACTIVE HYBRIDIZATION. Arnaud Huvet, Amaury Herpin, Jean-François Samain, and Charlie Cunningham, Laboratoire de Physiologie des Invertébrés, Ifremer, Centre de Brest, 29280 Plouzané, France, E-mail: ahuvet@ifremer.fr. and Sars International Centre for Molecular Marine Biology, Thormøhlensgt.55 N-5008 Bergen, Norway.

This project is part of the national multidisciplinary program "MOREST" recently begun in France with the aim of understanding the cause of summer mortality of juvenile *C. gigas*. Within this program, divergent selection criteria were applied at the Ifremer hatchery in La Tremblade (France). G2 progenies can be separated into two groups, R (for "Resistant") and S (for "Sensitive"), depending on their summer survival rates. Suppression subtractive hybridization (SSH) was conducted between R and S groups in order to identify the differential expression of key genes and dif-

ferences in physiologic function(s). Oysters from 3 R and 3 S groups were sampled during a mortality event in experimental conditions (summer temperature, reproductive period). Mortality in the 3 S groups was 80% whereas in the 3 R groups placed in the same tank it was less than 5%. The SSH library was generated using the Clontech PCR-select cDNA subtraction kit using mRNA from mantle-gonad tissues from a mix of 10 individuals from each R and S group.

Three hundred and seventy-six clones were obtained and screened on blots. One hundred and forty-eight clones (40%) appeared to be differentially expressed and were induced in the R group compared to the S group. These 148 clones were sequenced: 25% matched with the products of known genes; 6% matched hypothetical proteins; 9% displayed open reading frames of significant length but whose product was unknown and 40% appeared to be non coding sequences. Redundancy was approximately 20%. The SSH sequences that matched with known genes were clustered into 7 categories (Fig. 1).

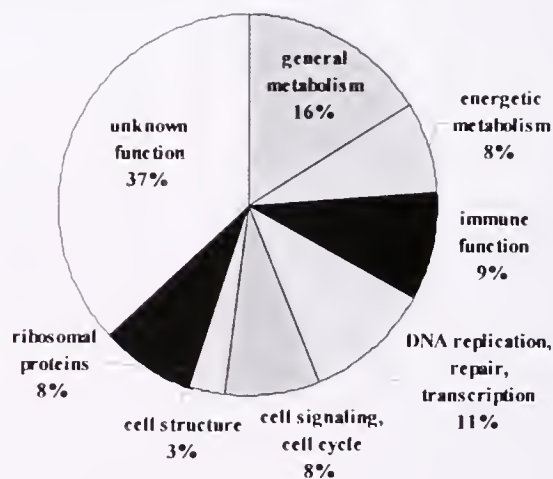


Figure 1.

The SSH sequences that appeared as interesting candidates to investigate summer mortality were fully sequenced. Full-length cDNA were obtained for 8 clones. These transcripts encoded homologues of the following proteins: cavortin, a self-aggregating haemolymph protein. cyclophilin, accelerates the folding of proteins and is secreted in response to stress stimuli. isocitrate dehydrogenase, implicated in the cellular defense against singlet oxygen-induced oxidative damage. scavenger receptor, a cell surface protein recognizing microbial constituents on the hemocyte surface. precerebellin, a known immune acute phase protein. sodium glucose cotransporter. fatty acid binding protein. ATPase, H^+ transporting lysosomal protein, involved in ATP deg-

radation, acidification of intracellular compartments required for zymogen activation, receptor-mediated endocytosis.

For those mRNA, real time PCR analyses are in progress.

GROW-OUT FARM SKILL TRAINING FOR THE BLACKLIP PEARL OYSTER *PINCTADA MARGARITIFERA* IN POHNPEI, THE FEDERATED STATES OF MICRONESIA.

Masahiro Ito, College of Micronesia Land Grant Program, P.O. Box 1179 Kolonia, Pohnpei FM96941, The Federated States of Micronesia. E-mail: hiroito@mail.fm

As a part of a project called "Development of Pearl Aquaculture and Expertise in Micronesia" (the Project), the College of Micronesia Land Grant Program (COM) initiated a grow-out farm training program for the blacklip pearl oyster *Pinctada margaritifera* during the last quarter of 2001, using spat (juvenile pearl oysters) produced at the COM's pearl hatchery in Pohnpei, the Federated States of Micronesia. Because a major cultured black pearl farming occurs in the French Polynesia and Cook Island of the South Pacific, hatchery-based black pearl farming is yet to be seen in many countries except for a few countries like Australia, Indonesian, Japan, and Philippines. Grow-out technology for the hatchery-produced spat is now available in the Micronesian region, and the Project aimed at transferring pearl aquaculture technology immediately to Micronesia by developing local human resources for support and maintenance of the pearl industry once it is established. For this purpose, three demonstration grow-out farms were established for a skill training at two island communities in Pohnpei State: two at Parem Island with 16 (102 m in length) longline systems located one mile north from the hatchery; and one at Pakin Atoll with six longline systems located 20 miles west off Pohnpei main island. The training was not limited to the hands-on care of hatchery-produced spat, but also included all aspects of farm-related work, such as farming site selection, deployment and maintenance of longline system and farming equipment, wild oyster collection, preparation for pearl seeding (grafting), and post-grafting care. During the first phase of the project, the program focused on a "train-a-trainer" method and started with several locals from the island communities. Along with the progress of the Project's hatchery training and spat production, more trainees were recruited beyond the two island communities. The program has entered "the second generation training" by the first generation trainees since the last quarter of 2003. All the trainees were unemployed whose age ranged from youth to elderly locals and they were provided stipends according to their participation, skill level, and attitude toward work. More than 50 people have so far participated in this program. To exercise an important role in the regional extension and research and education programs, the project has already accepted several college and high school students for field studies and work-study programs. Elementary school children were also provided field excursions, and other local and overseas stakeholders such as business investors, diplomats, and sci-

entists visited the hatchery and the farms. This presentation describes the COM's skill training methodology of the ocean nursery culture and evaluates briefly its hatchery-based pearl farming technique.

HATCHERY TRAINING ON THE BLACKLIP PEARL OYSTER *PINCTADA MARGARITIFERA* IN POHNPEI, THE FEDERATED STATES OF MICRONESIA.

Masahiro Ito, College of Micronesia Land Grant Program, P.O. Box 1179 Kolonia, Pohnpei FM96941, The Federated States of Micronesia. E-mail: hiroito@mail.fm

A hatchery-based farming is considered to be the best option to develop a pearl industry in the Micronesian region where wild resource of blacklip pearl oyster *Pinctada margaritifera* is not abundant like countries in the South Pacific region. It is unfortunate, however, that only a handful of private companies in Australia, Japan, and Philippines have been successful in such a hatchery-based black pearl farming. Hatchery technology for this species has not readily been available in this region until recently (Ito et al. 1995, Ito et al. 1996, Lucas et al. 1995, Southgate and Beer 1997, Southgate and Ito 1998, and Southgate et al. 1998). The College of Micronesia Land Grant Program (COM) built a small low-tech hatchery at Nett Point, Pohnpei during the third quarter of 2001 to implement an intensive training program. Funding was provided by the United States Department of Agriculture (USDA) and Department of Interior's Office of Insular Affairs (DOI) in support of this search for the pearl technology under a project called: "Development of Pearl Aquaculture and Expertise in Micronesia" (the Project). Three Micronesian trainees were recruited, who neither had background in pearl industry nor aquaculture experience and higher education; former government office clerk, a former agriculture extension agent, and an unemployed from one of the outer islands in Yap State. The Project aimed to transfer pearl hatchery expertise immediately to the Micronesians and to create core technicians within a limited timeframe of 2 to 3 years of intensive hands-on skill training, which will become a vehicle for development of hatchery-based pearl farming in this region.

The Project has been proving its efficiency and accomplished its first phase objectives on hatchery training, involving 31 series of spawning trials and seven larval runs, during the phase 1: (1) handling of all aspects of hatchery-related skills, including equipment preparation and system maintenance, microalgae culture, spawning induction, larval rearing, and spat culture; (2) sampling and data collection on feeding, larval development, and spat growth for basic scientific studies; (3) spat production for commercial-scale demonstration farms; and (4) handling all aspects of grow-out culture of hatchery-produced spat. The Project provided the first step towards an alternative approach to establish pearl industry in Micronesia by transferring a hatchery-based farming technology. This presentation describes the COM's methodology on pearl hatchery training and to evaluate briefly such techniques

as spawning induction, larval rearing, and in-tank spat culture of the blacklip pearl oyster.

DEVELOPMENT OF PEARL AQUACULTURE AND EXPERTISE IN MICRONESIA. Masahiro Ito, Robert Jackson, and Singeru Singeo. College of Micronesia Land Grant Program, P.O. Box 1179 Kolonia, Pohnpei FM96941, Federated States of Micronesia. E-mail: hiroito@mail.fm

Pearl industry in Micronesia has potential of becoming a major source of export income once it is developed. French Polynesia in the South Pacific alone exported over 100 million US dollars worth of cultured black pearls. Other Pacific countries are actively trying to develop their industry following the lead of French Polynesia and Cook Islands. Micronesian nations are far behind these South Pacific island nations in the development of production of its pearl industry. One of the reasons for this is the fact that there is not sufficient number of the blacklip pearl oysters that could be collected from the wild to supply pearl farms on a regular basis.

In 2001, the College of Micronesia (COM) embarked on a search for technology for production of pearl oyster spat in order to get around this lack of naturally occurring wild spat supply in Micronesian region. Funding was provided by the United States Department of Agriculture (USDA) and Department of Interior's Office of Insular Affairs (DOI) in support of this search for the pearl technology under a project called: "Development of Pearl Aquaculture and Expertise in Micronesia" (the Project). The general purposes of this project are to provide training programs for: (1) development of the pearl industry in Micronesia and (2) development of local human resources for supporting and maintaining the pearl industry once it is established. The funding support enabled the Project to commence its phase 1 activity during the first quarter 2001 and continued until the third quarter of 2003. All phase 1's objectives were accomplished: an Australian expert in pearl oyster hatchery technology was hired; a hatchery was established in an abandoned dock warehouse at Nett Point, Pohnpei; and the pearl expert and his Micronesian staff/trainees successfully conducted hatchery and ocean nursery events during the phase 1, resulting in tens of thousands of blacklip pearl oyster that are growing at the three demonstration farms, also established by the Project. Three Micronesian staff members are being trained as future trainers in spat production and farm grow-out technology, with participation of more than 50 trainees from local communities, schools, and colleges.

The Project entered phase 2 in the fourth quarter of 2003, proceeding to complete the necessary evaluations and demonstrations to the critical issues in the pearl industry development, including actual pearl production and business development. The phase 2 includes: (1) expansion of training of hatchery and grow-

out farm techniques, the second generation skill training by the Micronesian trainers; (2) implementation of pearl production trials and evaluation of seeding and pearl production techniques; and (3) collaboration with institutions in the region and government agencies in developing business models for the Micronesian pearl industry.

GROWTH AND SURVIVAL OF HATCHERY-PRODUCED SPAT OF THE BLACKLIP PEARL OYSTER *Pinctada margaritifera* DURING THE OCEAN NURSERY CULTURE IN POHNPEI LAGOON, THE FEDERATED STATES OF MICRONESIA. Masahiro Ito, Martin Hagilmai, and Justino Smith. College of Micronesia Land Grant Program, P.O. Box 1179, Kolonia Pohnpei, FM96941, The Federated States of Micronesia. E-mail: hiroito@mail.fm

Spat of the blacklip pearl oyster *Pinctada margaritifera* were produced from the hatchery built by the College of Micronesia Land Grant Program (COM) at Nett Point in Pohnpei, the Federated States of Micronesia. On Day 44 after fertilization, the spat attached to "in-tank spat collectors" were transferred to the grow-out farms, which were also built by COM. The growth and survivorship of the hatchery-produced spat were monitored during the ocean nursery culture. The spat were protected by spat bag (0.75 mm by 1.5-mm mesh) and suspended from a surface long line system at 2–3-m depth. After 2 months of initial ocean nursery culture, they were removed from the collectors and sorted into 48-pocket (4-mm square mesh) nets. At 4, 6, and 9 months of the culture, they were further sorted and transferred into 24-pocket (20-mm mesh) net, 15-pocket (30 mm mesh) net or lantern (9-mm mesh) net, respectively.

The spat grew to the mean (\pm SE) antero-posterior shell length (APL) and dorso-ventral shell height (DVH) of 38.0 (\pm 7.1) mm and 39.8 (\pm 6.4) mm, 57.4 (\pm 6.2) mm and 55.5 (\pm 7.1) mm, and 81.9 (\pm 8.1) mm and 83.9 (\pm 7.6) mm at 6 months ($n = 206$), 9 months ($n = 87$), and 12 months ($n = 118$) of the ocean nursery culture, respectively. The size of hatchery-produced spat after 184 days (6 months) of the ocean nursery culture in Pohnpei were similar to those at 196 days reported by Southgate and Beer (1997) from the northeastern Australia. Compared to the best growth in Australia given by Southgate and Beer (2000) for the hatchery-produced juveniles between 7 months (DVH 41.5 \pm 0.6 mm; $n = 40$) to 12 months (DVH 65.8 \pm 1.0 mm), those juveniles in Pohnpei grew faster from 6 to 12 months reaching to 83.9 \pm 7.6 with a maximum DVH recorded 97.6 mm. Survival rates from 4 to 12 months and 6 to 12 months in Pohnpei were 83.2% and 91.3%, respectively ($n = 6384$ at 4 months; $n = 5814$ at 6 months; and $n = 5310$ at 12 months). Ongoing hatchery and ocean nursery trials with several batches since the first quarter of 2002 revealed that the survival rates were constantly high during the monitoring periods from 4 to

6 months, 6 to 9 months, and 9 to 12 months, which scored between 88.9%–98.5%. Hatchery-produced juveniles also showed uniformity in their shape, example, average DVH/APL (\pm SE; n) ratios of the first and second batches from the hatchery runs in 2002 were 1.035 (\pm 0.050; $n = 118$) and 1.042 (\pm 0.085; $n = 139$) at 12 months of nursery culture, respectively, and there was no significant difference ($P > 0.05$) between their average ratios. These findings suggest that our grow-out culture methodology is proving its efficiency and the farming environment of the Pohnpei lagoon in Micronesia is well suited for growing hatchery-produced *P. argaritifera*, and could have a potential for producing high quality black pearls also. Nuclei implantation commences from September 2003 to conduct pearl quality experiments using the hatchery-produced and wild-caught pearl oysters.

CAWTHRON'S ULTRA DENSITY LARVAL SYSTEM (CUDLS) FOR REARING LARVAE OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS*. Achim Janke, Henry Kaspar, Nick King, and Sam Foster, Cawthron Institute, Private Bag 2, Nelson, New Zealand. E-mail: achim.janke@cawthron.org.nz

Conventional commercial systems for rearing bivalve larvae use large tanks with water exchanged every 2 to 3 days and larval densities of 5 to 10 larvae per mL at the start of larval rearing reducing to 1-eyed larva per mL just prior to settlement. Heating the large volume of seawater required for larval rearing incurs major capital and operating costs. Continuous exchange systems offer the potential to significantly reduce these costs.

All bivalve larval rearing systems require water exchange to prevent build up of metabolic wastes and subsequent degradation of water quality. In a static system, water quality is at its best immediately after water exchange and degrades progressively until the next water exchange. In a continuous system, water is exchanged constantly ensuring minimal fluctuation in water quality and allowing an increase in larval density. In CUDLS, the increase in larval density has resulted in water savings of up to 45% and a reduction in tank volume by 99% over the equivalent static system. Larvae have been reared for their first week at densities of over 1000 per mL without detrimental effects on growth or survival. Commercial production using this system has yielded over 35 million eyed larvae ($>350 \mu\text{m}$) after 18 days at 22 °C from two 170-L tanks with each tank exchanging seawater at 3.5 L per minute.

Larvae are fed mainly *Chaetoceros calcitrans* and *Isochrysis galbana* Tahitian strain (T-iso), with feeding rates determined by observation of larval condition and algal clearance rates. As a guide larvae are fed the equivalent of 80 *Isochrysis* cells per μL at the start of larval rearing and in excess of 200 *Isochrysis* equivalent cells per μL just prior to settlement.

SELECTIVE BREEDING OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS* IN NEW ZEALAND. Achim Janke, Nick King, Rodney Roberts, and Henry Kaspar, Cawthron Institute, Private Bag 2, Nelson, New Zealand. E-mail: achim.janke@cawthron.org.nz

The Pacific oyster was accidentally introduced to New Zealand in 1971 and farming of this species now forms the basis of an industry worth 15 million USD per annum. Until recently, most oyster production in New Zealand was derived from wild caught spat. With the move toward single seed culture of hatchery produced spat, the opportunity exists for commercial growers to achieve genetic gain through selective breeding.

A Pacific oyster-breeding program was begun by the Cawthron Institute in 1999. The program uses randomly selected wild oysters as parents for the first generation of full-sib families, with recurrent selection both within and between families for subsequent generations.

A trial cohort of nine randomly mated full-sib oyster families was produced in 1999. After 5 months of grow-out significant differences ($P < 0.01$) in growth rate were found among families. After 10 months 95% of the oysters had reached marketable size. The fastest growing family was more than twice as heavy as the slowest growing family, and 25% heavier than the average. Significant differences in growth rate existed among the four grow-out sites ($P < 0.01$), but no interaction was identified between growth rate and site ($P > 0.05$) meaning that family rankings for growth rate were consistent across a range of culture methods and growing environments. Survival averaged 85% across the four sites. These preliminary results show that differences among families are significant and that considerable gains in growth rate can be achieved through selective breeding.

A second cohort of 60 full sib oyster families was produced in 2001. These oysters reached marketable condition in 2003 and work on assessing these families is underway. The best oysters from the top performing families in this cohort will be selected for use as parents of a second generation.

IMPACT OF THE REPRODUCTION DYNAMICS ON GENETIC VARIATION IN THE EUROPEAN FLAT OYSTER *OSTREA EDULIS*. Sylvie Lapègue, Nicolas Taxis, Delphine Lallias, François Bonhomme, and Pierre Bondry, IFREMER, Laboratoire de Génétique et Pathologie, Ronce Les Bains, 17390 La Tremblade, France. E-mail: slapegue@ifremer.fr

The European flat oyster (*Ostrea edulis* L.) is a marine bivalve whose natural geographical distribution ranges along the European Atlantic coast from Norway to Morocco, in addition to the Mediterranean and Black Sea. The latest results obtained on the genetic differentiation between these populations have led us to

pursue studies at a finer scale, in order to estimate the effective number of breeders and the temporal dynamics of reproduction and, more specially, recruitment. Several experiments were performed to document (1) the variance in allele frequencies during a natural settlement period; (2) the paternal contribution to fertilization by analyzing larvae sampled at the brooding stage within individual females; (3) the variance of individual reproductive success within an experimental population.

Firstly, 3 sets of collectors were successively deployed every 2 weeks and one set left during the whole recruitment period in 2001. In addition, adult oysters were sampled including 14 brooding females (ie, females presenting larvae in their paleal cavity). Their larvae were sampled and preserved in ethanol. Mitochondrial (12S fragment) and microsatellite (four loci) analyses were performed.

Although the temporal cohorts did not exhibit any differentiation on the basis of the microsatellite markers, a slight but significant differentiation was observed with the mitochondrial marker. Moreover, our data on the genetic variability of single-female progenies show that females can be fertilized by a highly variable number of males, which can be, in some cases, very low (Fig. 1). In such cases, a temporary low effective could lead to a level of inbreeding (even low) which would explain the correlation between growth and heterosis often observed.

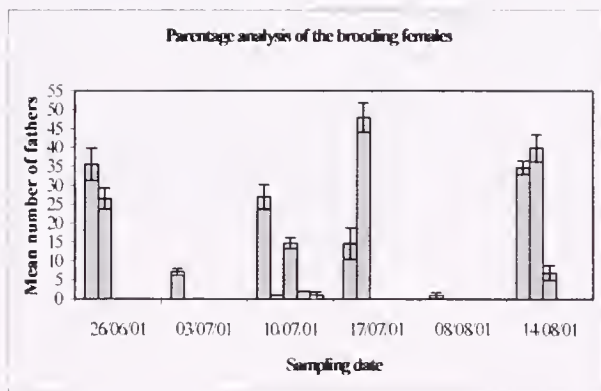


Figure 1.

More investigations are needed to directly demonstrate that each male fertilizes several females. If males succeed in fertilizing several females, variability in environmental conditions should increase female variance in reproductive success more than male variance in reproductive success, thus reducing mitochondrial relative to nuclear effective size as has been observed in the study of the genetic structure of the populations along the species range.

COMPARISON OF FIVE REARING TECHNIQUES FOR THE GIANT SCALLOP *PLACOPECTEN MAGELLANICUS* IN THE GASPÉ BAY, QUEBEC, CANADA. Marie-Lyne Larrière, Laurent Girault, Fabrice Pernet, and Benoît Thomas, Centre collégial de transfert de technologie des pêches, 167 La Grande Allée Est, C.P. 220, Grande-Rivière, Québec, Canada G0C 1V0. E-mail: ml_larriere@globetrotter.net

The giant scallop is an indigenous species of the Gaspé Peninsula, which represents high commercial value; therefore it is potentially interesting. Several rearing techniques are available and it is preferable to determine which is the most adapted to the particular conditions of the Gaspé Bay before undertaking commercial culture.

Juveniles were therefore imported from Magdalene Islands in spring and autumn of 2001 in order to carry out this evaluation. They were immersed into the Gaspé Bay at three different depths into five kinds of devices: earrings (ER), pearl nets (PN), Wang-Joncas lanterns (WJ), Savoury cages (SC) and oyster tables (OT). Growth rates, mortalities and several environmental variables are being followed for each depth, each device and each season of transfer until 2004. Biochemical analyses will supplement field measurements.

Mortalities were high for the scallops transferred during spring, varying, according to device, from 59%–82% (Fig. 1). These losses are mainly ascribable to the combined stresses of transfer and spawning. Mortalities approached normal values after the autumnal transfer. Growth rates are comparable with those observed elsewhere in Quebec (Fig. 2). They are similar for ER, PN, and SC, but they are lower for OT and WJ. The effect of depth is more noticeable on the meat yield, which is higher near the surface, than on the shell growth rate.

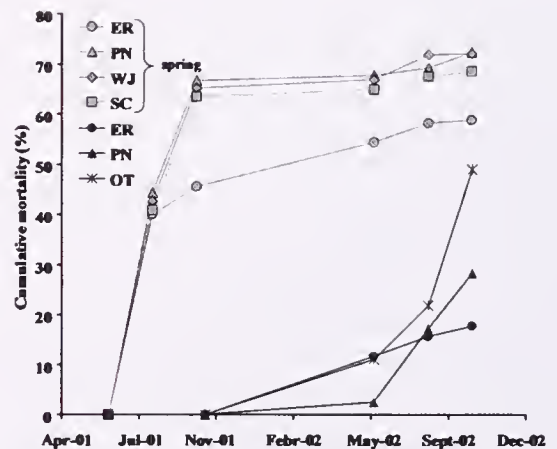


Figure 1. Scallops cumulative mortality according to the rearing device.

Because Gaspé Bay presents significant interannual variations of the environmental parameters, the later follow-ups will be use-

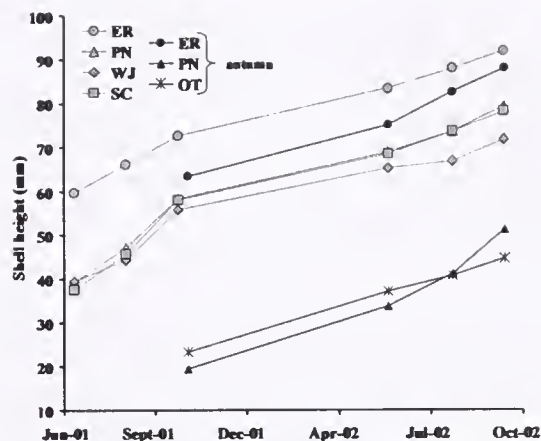


Figure 2. Evolution of scallops average sizes according to the rearing device.

ful to determine the average and extreme conditions that consequently will optimize the rearing strategy. In addition, it is increasingly evident that the meat yield, which can only be determined at the end of the growth cycle, is the important factor in the determination of economic feasibility of each method.

POPULATION GENETICS OF THE BLACK-LIP PEARL OYSTER *PINCTADA MARGARITIFERA*. Teresa Lewis, Candace Martin, Cameron Muir, Maria Haws, Simon Ellis, Matang Ueanimatang, Donald David, and Manoj Nair, Hawaii Institute of Marine Biology, School of Ocean and Earth Sciences and Technology, University of Hawaii at Manoa, Kaneohe, Hawaii. E-mail: tdlewis@hawaii.edu

Pearl production is an important industry that promotes sustainable economic development and provides export income for various Pacific Island nations. Empirical evidence points towards a significant difference between pearls produced by different stocks of pearl oysters. For example, the island of Manihiki in the Cook Islands produces recognizably distinct pearls with unique coloration. Pearl buyers in French Polynesia noted that before large-scale pearl oyster transfers took place between the dozens of atolls in the Tuamotu Islands chain, atolls tended to produce pearls with recognizable differences in color, luster, and orient, critical factors in establishing price and a competitive niche. After massive stock transfers of spat between islands occurred, these island-specific differences disappeared. There is a need to develop sensitive, and accurate genetic fingerprints to facilitate monitoring and development of appropriate management practices in black-lip pearl oyster aquaculture. Hatchery operators require information to formulate strategies that will allow them to supply stakeholders with the requested spat while protecting biodiversity and the potential economic value related to genetic difference between

stocks. We are seeking to address this issue through the use of two DNA marker systems: amplified fragment length polymorphism and analysis of microsatellite DNA. Samples included in our analyses were collected from hatcheries in Hawaii, the Federated States of Micronesia, and the Republic of the Marshall Islands, and from natural stocks. Results from the combined outcomes of these two avenues of research will be presented.

EXPRESSION LIBRARY IMMUNIZATION FOR DEVELOPMENT OF VACCINES FOR USE IN AQUACULTURE.

Teresa D. Lewis, Ichiro Misumi, Christopher D. Kelley, Michael L. Kent, and Jo-Ann C. Leong, Hawaii Institute of Marine Biology, School of Ocean and Earth Sciences and Technology, University of Hawaii at Manoa, Kaneohe HI, USA. E-mail: tdlewis@hawaii.edu

Various techniques have been applied for the development of vaccines against fish pathogens that impact aquaculture. For viruses, this has met with the most success by developing a vaccine against an immunogenic epitope in the viral coat glycoprotein. For bacteria, bacterins and sub-unit vaccines have been the most efficacious. Fish parasites generally have multiple life stages and the cellular immune response may contribute to a more successful protective response than the humoral response. Thus, development of vaccines against parasites has proven more problematic. Here we describe the technique of expression library immunization (ELI) and the application of this method for identifying immunogenic proteins of *Cryptocaryon irritans*. The usefulness of this method over other forms of DNA vaccination is its application against pathogens for which we have no *a priori* knowledge of the proteins encoded by the genome. An acquired protective immune response against this parasite has been observed in thick-lipped mullet, mummichog minnow, and barramundi that were immunized by controlled infections followed by challenge infection. Here we report on our progress in screening a *C. irritans* genomic expression library to identify immunogenic proteins suitable for use in vaccine development.

MARINE AGGREGATES AS RESERVOIRS FOR THRAUSTOCHYTRIDS SUCH AS THE BIVALVE PARASITE, QUAHOG PARASITE UNKNOWN (QPX). M. M. Lyons, J. E. Ward, Department of Marine Sciences, University of Connecticut, Avery Pt., CT, USA; K. R. Uhlinger, and R. Smolowitz, Marine Resource Center, Marine Biological Laboratory, Woods Hole, MA, USA.

Marine aggregates (eg, marine snow, organic detritus, and flocs) may enhance the transmission of shellfish diseases by at least two mechanisms: (1) as reservoirs, if aggregates concentrate

pathogens within their matrix; and (2) as vectors, if pathogen-laden, marine aggregates are more readily captured and retained by the gills of suspension-feeding bivalves. Northern hard clams (= quahog; *Mercenaria mercenaria*) from the northeast coast of North America have suffered severe mortalities from a bivalve parasite known as quahog parasite unknown (QPX). QPX is a facultative parasite but little is known regarding the ecology of QPX. Morphologic and molecular analyses have characterized it as a thraustochytrid. Thraustochytrids are single-celled, eukaryotic, fungal-like, marine protists associated with decaying vegetation, shells, and marine aggregates. The focus of this research is: if QPX is a thraustochytrid, and thraustochytrids are linked to marine aggregates then, are marine aggregates one of the reservoirs for QPX? Detection strategies for QPX-enriched marine aggregates, including fluorescent stains and *in situ* hybridization, will be presented along with endoscopic video illustrating hard clams filtering marine aggregates. Hard clams ingest some, but not all, aggregates. Those that are not ingested are collected at the base of the incurrent siphon until rejected as pseudofeces. Because this is also the site of QPX-filled nodules in infected clams from Massachusetts, we hypothesize marine aggregates facilitate infection.

POSTINGESTIVE SELECTION IN THE SEA SCALLOP *PLACOPECTEN MAGELLANICUS* ON THE BASIS OF PHYSICAL AND CHEMICAL PROPERTIES OF THE PARTICLES. Bruce A. MacDonald and Martha G. S. Brillant, Biology Department and Centre for Coastal Studies and Aquaculture, University of New Brunswick, Saint John, N.B. Canada E2L 4L5. E-mail: bmacdon@unbsj.ca

Suspension-feeding bivalves may enhance the energy value of their food supply by sorting particles before and after ingestion. Postingestive sorting in bivalves has been confirmed for several species, but few studies have attempted to isolate the factors influencing postingestive selection among different particles presented simultaneously. In a series of experiments sea scallops were exposed to (1) beads of different sizes and density to determine the importance of physical factors; (2) ^{14}C -labeled dinoflagellates and ^{51}Cr -labeled beads of similar size and protein-coated and uncoated beads of identical size but different colors; and (3) living and heat-killed *Chlorella* with different chemical composition to assess the influence of particle chemical characteristics. Comparisons of gut retention times demonstrated that *P. magellanicus* can distinguish between particles of different sizes and densities, retaining larger particles (20 μm) longer than smaller ones (5 μm) and lighter particles longer than denser ones. The ^{14}C : ^{51}Cr ratio in the stomach decreased over time, indicating that the scallops were sorting organic from inorganic particles. Flow cytometry studies showed that scallops retained protein-coated beads longer than uncoated beads indicating simultaneous postingestive selection based solely on chemical properties. *P. magellanicus* retained live *Chlorella* cells longer than nutritionally poorer heat-killed cells

demonstrating this species's ability to distinguish between two physically identical but nutritionally different forms of the same species of microalgae. These experiments have confirmed the ability of sea scallops to sort particles within the gut based on the physical and/or chemical properties. This capability will enable the sea scallop to efficiently process a diet that is extremely variable in quality and enhance the energy uptake while minimizing losses associated with digestion.

A COMPARISON OF DREDGE AND PATENT TONGS FOR ESTIMATION OF OYSTER POPULATIONS. Roger Mann, Melissa Southworth, and Juliana M. Harding, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA; and James Wesson, Virginia Marine Resources Commission, P.O. Box 756, Newport News, VA 23607, USA.

Exploited oyster stocks on public grounds in Virginia waters are subject to regular surveys effected using a traditional oyster dredge and, more recently, patent tongs. Dredges provide semi-quantitative data, have been used with consistency over extended periods (decades), and thus provide data on population trends. Patent tongs provide absolute quantification but are more labor intensive. Absolute quantification of dredge data is difficult in that dredges accumulate organisms as they move over the bottom, may not sample with constancy throughout a single dredge haul, and may fill before completion of the haul, thereby providing biased sampling. Selectivity of dredges versus patent tongs with respect to demographics has not been rigorously examined. The objective of this study is to compare data from both sampling protocols. Data for the study were taken from 1998 to 2001 surveys conducted in the James River, Virginia by the Virginia Institute of Marine Science and the Virginia Marine Resources Commission wherein the same stations were sampled by both techniques. Dredge surveys give data in oysters per bushel and assume no selective retention of live oysters with respect to shell substrate by the dredge. Data from patent tongs is archived as per tong estimates of oysters by size class and shell by volume. The hydraulically operated 1-m square tong used in VMRC/VIMS surveys is designed to sample on and below the reef surface, which includes elements of buried shell that are probably not well sampled by a dredge, although the sampling insures collection of all oysters within the tong mouth. Database entries were pooled with oysters <25 mm being distinguished as spat, the 25–75 mm being distinguished as sub market or small oysters, and >75 mm as market oysters, then bushel estimators applied as described earlier. This allowed estimation of both total volume and #/bushel, the unit given by dredge data.

The summary plot of mean values from 1998 to 2001 illustrates differences in the sampling gear. More shell per unit oyster (lower bushels counts) are found in a tong sample. The appropriate model for attempting to fit a predictive line is open to debate, and will be influenced by patent tong penetration as determined by the degree

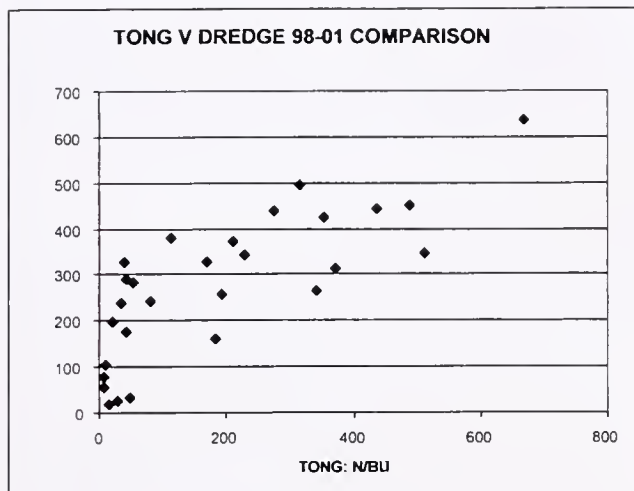


Figure 1.

of consolidation of the underlying substrate. The data do not strongly support the ability to predict a relationship between dredge and patent tong population estimates at this time.

PERKINSUS MARINUS INFECTION RATES & PROGRESSION IN POPULATIONS OF SPECIFIC-PATHOGEN-FREE JUVENILE EASTERN OYSTERS *CRASSOSTREA VIRGINICA* PLANTED IN A TRIBUTARY OF CHESAPEAKE BAY. Carol B. McCollough, Christopher F. Dungan, Mark Homer, George R. Abbe, and Candace A. Morrellm, Maryland Department of Natural Resources, Cooperative Oxford Laboratory, Oxford, MD 21654, USA. E-mail: cmccollough@dnr.state.md.us

Specific-pathogen-free (SPF) oysters were set and reared in artificial seawater and transferred to four sites in the Patuxent River along a putative salinity gradient. Three sites were adjacent to natural oyster bars and one was remote from existing oyster populations. Deployments were made in September 2000, June and August 2001, and May and September 2002. Samples of 30 oysters were assayed at 2 and 4 weeks post-deployment for infection by *P. marinus* using an enhanced RFTM whole body burden technique. Assays continued at 4-week intervals until infections were detected in 3 consecutive samples, except the initial 2000 deployment was followed until 100% mortality occurred.

During drought conditions, SPF juvenile oysters acquired *P. marinus* infections as early as 2 weeks post-deployment. Infection rates declined markedly during freshet conditions (winter/spring 2003). SPF juvenile oysters placed at sites remote from natural oyster populations harboring *P. marinus* acquired infections at similar rates as SFP juveniles adjacent to natural populations. Both infection prevalences and mortalities demonstrated similar seasonal patterns.

EFFECTS OF MICROALGAL DIETS AND FATTY ACID COMPOSITION ON THE GROWTH PERFORMANCE OF POSTLARVAL AND JUVENILE BAY SCALLOPS *ARGOPECTEN IRRADIANS*. Lisa M. Milke, V. Monica Bricelj, and Christopher C. Parrish, National Research Council, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, NS B3H 3Z1 Canada, and Department of Biology, Dalhousie University, Halifax, NS B3H 4J1 Canada. E-mail: lisa.milke@nrc.ca

Hatcheries often rely on multi-species algal diets for the production of postlarval and juvenile scallops, because nutritional requirements have not been adequately described. However, due to high costs of algal culture there is an incentive to reduce the number of species utilized. The goals of our study were: (1) to identify high-performance diets, comprised of a minimal number of species, that will maximize production of bay scallop postlarvae and juveniles and (2) to examine potential differences in the nutritional requirements, especially of lipids, at different developmental stages. To this end, postlarval (initial shell height, SH = 240 μ m) and juvenile (initial SH = 10 mm) bay scallops were offered 6–7 microalgal diet combinations at 20 °C, for 3 weeks.

Superior growth rates for both developmental stages were attained on a diet of *Pavlova* sp. (CCMP 459) and *C. muelleri* (CHGRA). This diet is characterized by elevated levels of n-6 fatty acids, arachidonic (AA) in CHGRA and docosapentenoic (DPA) in Pav 459, which may contribute to the high performance of this diet. In fact, the two diet treatments that were deficient in AA and DPA, *P. lutheri*/*T. weissflogii* and *P. lutheri*/*F. famolica*, yielded the lowest growth rates at both developmental stages. These results, in conjunction with our previous work on sea scallop, *Placopecten magellanicus*, postlarvae suggests a dietary need for n-6 fatty acids across pectinid species.

The *T. striatal*/CHGRA combination, which is limited in docosahexaenoic acid (DHA), was the second highest performing diet in both stages of bay scallops although it is unsuitable for sea scallop postlarvae. Overall, limited enrichment of DHA was observed in the tissues relative to the diet, although there was a marked increase in tissue DHA/EPA ratios compared to the diets. This may be attributed to a decrease in EPA, possibly through selective catabolism and/or modification as has been previously suggested for *Argopecten purpuratus* larvae. This suggests that bay scallops may have less stringent dietary DHA requirements than sea scallops, and may be similar to oyster spat, *Crassostrea gigas*, which require either DHA or EPA, but not both, to sustain growth.

The *Rhodomonas lens*/CHGRA combination was the only diet exhibiting stage-specific differences in growth rate: it was a moderate diet for juveniles but poor for postlarvae. However, five of the six diets tested showed no difference in growth performance between stages, suggesting that in relation to growth performance, bay scallop postlarvae and juveniles have similar nutritional requirements.

DENSITY SEPARATION OF RECENTLY SETTLED MANILA CLAMS *TAPES PHILIPPINARUM* FROM THREE TYPES OF SEDIMENT. D. Munroe, D. Bright, and S. McKinley, Center for Aquaculture and the Environment, Faculty of Agricultural Sciences, University of British Columbia, 4160 Marine Dr., West Vancouver, BC, V7V 1N6. E-mail: dmmunroe@interchange.ubc.ca

Field studies are essential for the adequate study of larval biology and recruitment. Field study of the early juvenile life stages of wild intertidal clam populations is difficult because enumeration of bivalves in sediments is tedious and the accuracy is unknown. Therefore, a simple and effective method is needed to easily and accurately sample and count early juvenile bivalves from intertidal sediments. Here, we test the efficacy of methods for separation of recently settled Manila clams (*Tapes philippinarum*) from different types of intertidal sediments using density. Three aquaria were set up with sterilized sediments of each of the three sediment types: cobble/sand/shell, cobble/mud, mud/sand (9 tanks total), and each was filled with filtered, sterilized sea water heated to 20 °C and aerated. A known number of competent *T. philippinarum* larvae were added to each tank and allowed to settle and metamorphose, then the aquaria were drained and four sediment samples were taken from each aquarium for evaluation of number of juvenile clams present. Samples were wet sieved to isolate the size fraction from 125–500 µm, then this size fraction was settled through high density (1.9 g/mL) sucrose solution to separate stained clams from sediments. The average numbers of clams counted per sample for each sediment type were: 57.9 (SD = 37.1) for mud/sand, 60.0 (SD = 36.1) for cobble/sand/shell, and 53.1 (SD = 39.6) for cobble/mud (Fig. 1).

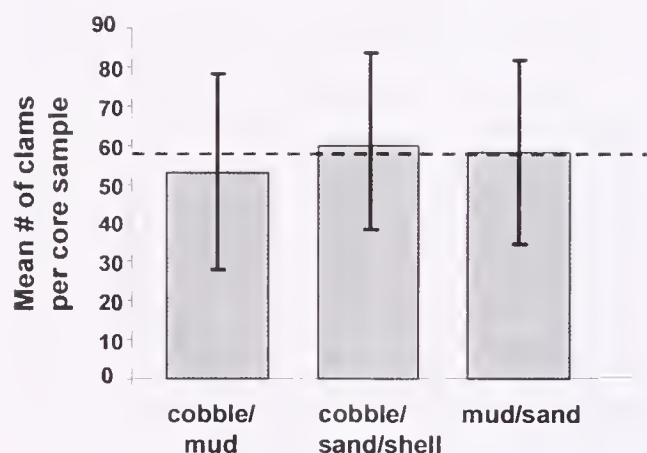


Figure 1. Means and 95% confidence limits for numbers of clams per sample for the three sediment types. The dashed line indicates the expected number of clams per sample (58.8) based on number of larvae placed in each tank. $N = 12$ for each treatment.

Based on the number of larvae originally added to each tank, the average number of clams expected per sample was 58.8 for all

three sediment types. There was no significant difference between the mean number of clams per sample and the expected value of 58.8 for all three sediment types. Therefore, these results indicate that the methods used here can be used to rapidly extract recently settled Manila clams from all sediment types with a high degree of accuracy.

STATUS OF BLACK LIP PEARL OYSTER FARMING IN THE REPUBLIC OF THE MARSHALL ISLANDS. Manoj R. Nair and Rand Dybdahl, College of the Marshall Islands, PO Box 1258, Majuro, MH96960, Republic of the Marshall Islands. E-mail: manojnair999@yahoo.com

Pearl farming is one of the important sources of revenue for several Pacific Island nations, including the Republic of the Marshall Islands (RMI). This aquaculture venture has been one of the main aquaculture revenue earners after the giant clam mariculture. The black lip pearl oyster *Pinctada margaritifera* is also available in some of the selected atolls of the RMI where they are being exploited for commercial purposes. There are 4 successful pearl farms in the country and they are in the process of expansion and several new entrepreneurs have also shown keen interest in pearl farming. However the major bottleneck for the successful and sustainable pearl farming in the region is the non-availability of sufficient wild pearl stocks. Earlier studies have shown that the natural stock in the RMI cannot support for the industry to go commercial in a sustainable manner and there is every possibility of the existing small stocks of pearl oysters being totally fished out. Experiments on wild spat collection in the different atolls have also given poor results. This problem has been overcome successfully recently with the development of commercial hatchery technology to produce spat for the industry. Seeing the importance and the potential of pearl farming as a primary or supplementary source of income to the community, the government is in the process of encouraging small-scale ventures as alternate income generation method to the traditional copra while simultaneously initiating steps on sustainable fisheries management strategies. The paper discusses the present status and the future pearl farming scenario in the RMI.

MUSSEL FARMING IN INDIA: PRESENT STATUS AND FUTURE PROSPECTS. R. Manoj Nair, USDA Land Grant, Cooperative Research and Extension, College of the Marshall Islands, PB-1258, Majuro, MH 96960, Republic of the Marshall Islands. E-mail: manojnair999@yahoo.com; K. K. Appukuttan, Molluscan Fisheries Division, Central Marine Fisheries Research Institute (CMFRI), PB-1603, Cochin 682014, Kerala, India; and T. S. Velayudhan, Molluscan Fisheries Division, Central Marine Fisheries Research Institute (CMFRI), PB-1603, Cochin 682014, Kerala, India.

India has two commercially important species of mussels. Green mussel *Perna viridis* and the Brown mussel *Perna indica*. In Indian literature green mussel was often described as *Mytilus viri-*

dis; this has been synonymized under *Perna viridis*. The former has a wide distribution along the Indian coasts and about 10,000 t of mussels are exploited yearly from the west coast of India, mainly from the state of Kerala. They support traditional sustenance fishing and scope of increasing natural production from existing beds is rather limited.

Mussel culture in India using *Perna viridis* and *Perna indica* was first conducted on an experimental scale in the early seventies by the Central Marine Fisheries Research Institute (CMFRI) at Calicut and Vizhinjam. Although the technology was developed by CMFRI in the mid and late seventies, it was only two decades later that the first commercial culture of mussels (Green mussel *Perna viridis*) was started (late 1995) at Anthakaranazhi (Alleppey District) in Kerala by local fishermen on long lines in the sea, with the technical support of CMFRI. Commercial mussel culture activity along the south west coast of India picked up in a big way since 1997 in different parts of Kerala in the open ocean and the estuaries lately by women groups, and Karnataka. Cultured mussel production is steadily increasing and it is expected to be about 1000 t in the year 2003; the production is projected to be 120,00 t by the year 2007 to 2008. Since the first experiments in the 1970s several new innovations have been tried. This paper discusses the evolution of mussel culture in India its development, present status, and its future prospects.

AN OVERVIEW OF PEARL FARMING IN INDIA. Manoj R. Nair, USDA Land Grant, Cooperative Research and Extension, College of the Marshall Islands, PB-1258, Majuro, MH 96960, Republic of the Marshall Islands. E-mail: manojnair999@yahoo.com; K. K. Appukuttan and T. S. Velayudhan, Molluscan Fisheries Division, Central Marine Fisheries Research Institute (CMFRI), PB-1603, Cochin 682014, Kerala, India.

India is well known for the production of beautiful natural pearls. India has a wealth of pearl producing oysters. The main ones among these are the Akoya oyster *Pinctada fucata* distributed from the famous Gulf of Mannar, Palk Bay, and Gulf of Kutch (mistakenly identified and wrongly reported by many even now as *Pinctada radiata*) and the Black lip pearl oyster *Pinctada margaritifera* in the Andaman and Nicobar Islands. Pearl culture in India was first conducted on an experimental scale in the early 1970s by the Central Marine Fisheries Research Institute (CMFRI) at its Tuticorin research center on the south eastern Coast of India. The institute had initiated experimental pearl production in 1972 and the first Indian cultured pearl produced the following year. Hatchery technology was developed by CMFRI for *P. fucata* and *P. margaritifera* in the early (1982) and late eighties (1987) respectively. With the technology, being standardized after repeated experimentation, sea farming of pearl oysters, cultured pearl production, hatchery production, etc was taken by private entrepreneurs and coastal community groups on both coasts of India pearl farm-

ing. This paper, in addition to giving an overview of evolution of the Indian marine pearl farming, also discusses the recent innovations like onshore pearl culture.

RECOVERY OF ENDANGERED MUSSEL POPULATIONS IN RIVERS OF THE EASTERN UNITED STATES. Richard J. Neves, Virginia Cooperative Fish and Wildlife Research Unit, US Geological Survey, 106 Cheatham Hall, Virginia Tech, Blacksburg, VA 24061, USA. E-mail: mussel@vt.edu

Of the nearly 300 species of native freshwater mussels in the United States, 35 are presumed extinct and 70 are federally listed as endangered or threatened. Because large mussels are capable of filtering roughly 40 L of water per day, historic mussel beds provided an ecologically important biofiltration function for most rivers in the central and eastern United States. Their filtration of phytoplankton, bacteria, and particulate organic matter from the water column provided an ecological service; namely, to enhance nutrient cycling and improve water quality for other species. The Freshwater Mollusk Conservation Center at Virginia Tech is now propagating and releasing endangered juvenile mussels of species in need of recovery. Initial releases of cultured juveniles began in 1997 and, since that time, roughly 100,000 endangered juveniles per year have been released into various river systems. The techniques and recirculating aquaculture technology now in use with rare species can be applied to any species in any river system of the country. Involvement in mussel propagation by state and federal facilities is now expanding to address the conservation needs of rare species and to produce juvenile mussels for toxicity testing, in order to assess whether water quality criteria are protective of these species. The restoration or augmentation of mussel populations in rivers provides another opportunity to improve water quality and sustain the health of freshwater ecosystems affected by anthropogenic stressors. Propagation of native freshwater mussels now provides a means to recolonize historic habitats and restore the natural biofiltration system of waterways throughout the United States.

BALLAN WRASSE *LABRUS BERGYLTA* PREDATION ON SCALLOP SPAT *PECTEN MAXIMUS*. G. G. Oppegård, T. Strohmeier, G. Bakke, O. Strand, I. Mayer, and A. Breistol, Department of Fisheries and Marine Biology, University of Bergen, High Technology Center, N5020 Bergen, Norway. E-mail: guri.oppegard@student.uib.no

Sea ranching of the great scallop (*Pecten maximus*) has been identified as having a high potential for future aquaculture in Norway. Until recently the edible crab (*Cancer pagurus*) and starfish (*Asterias rubens*) has been the major predators, causing great losses of released scallops. In order to reduce these losses a functional fence was designed to protect scallop sea ranches. The fence has improved the survival rate greatly.

The scallops are hatchery-reared and sold on to farmers at a size of 15–20 mm for on-growing. For protection against predation, the spat are grown in cages for the first 1–2 years before being seeded out onto the bottom. During this intermediate cage cultivation, the spat attain a size of 50 mm.

To reduce costs and avoid labor intensive cleaning associated with the intermediate culture, farmers are now seeding smaller spat into the fenced sea ranches.

During the summer of 2002 Helland Skjell AS, Norway, performed preliminary studies where spat (30–40 mm) were seeded from intermediate culture to bottom culture in a fenced site. Ballan wrasse (*Labrus bergylta*) were attracted to the area and the fish were observed to nab and eat on the spat. Studies on food preference for ballan wrasse performed in Ireland and France show various foraged species in stomach content where two other bivalves, mussel (*Mytilus edulis*) and *Chlamys* spp. are important prey.

The objective of this study is to find the maximum scallop size that the ballan wrasse can eat. This will provide information on the minimum spat size that can be released into fenced seabed avoiding heavy predation from ballan wrasse.

In experimental tanks (1 m³) the wrasse is given scallops in a size range of 15–35 mm, and foraged scallops are calculated after 1 week. Preliminary results indicate that the ballan wrasse do not eat scallop larger than 30 mm.

The first experiment is performed without bottom sediment. These results are compared with an identical experiment including sediment. A third aim for the study is to determine whether there are differences in survival success when the scallops are given time to recess in sediment before ballan wrasse is introduced into the tank, compared with a situation where spat are seeded in a tank with wrasse already present.

Results from these experiments will be presented.

ADVANCES IN THE CRYOPRESERVATION OF SPERM OF THE RED ABALONE *HALIOTIS RUFESCENS*. Carmen G. Paniagua-Chávez, Liliana Salinas-Flores, Miguel A. del Río-Portilla, and Rebeca Vásquez-Yeomans, Department of Aquaculture and Marine Biotechnology, Centro de Investigación Científica y Educación Superior de Ensenada, Ensenada, Baja California, México. E-mail: cpaniagu@cicese.mx

The demand for abalone throughout the world has increased dramatically and thus interest in cultivation has augmented. The culture of abalone in Baja California, México is a flourishing industry that requires supporting technologies. The cryopreservation of sperm of different species of abalone could be a good alternative, not only to solve conservation problems for endangered species, but also to improve the production of commercially reared cultures.

In previous years, different studies related to the cryopreservation of sperm of red abalone have been performed to optimize

protocols. Refrigerated storage (4 °C) of sperm and the effect of addition of antibiotics such as ampicillin, gentamicin, and antibiotic/antimycotic on the viability of sperm were evaluated. The use of antibiotics prolonged refrigerated storage of sperm and no significant differences were found on sperm motility after addition of antibiotics (Fig. 1). Moreover, bacteria growth was inhibited up to 90%. These results showed that refrigerated storage of red abalone sperm could be a helpful tool on hatchery management.

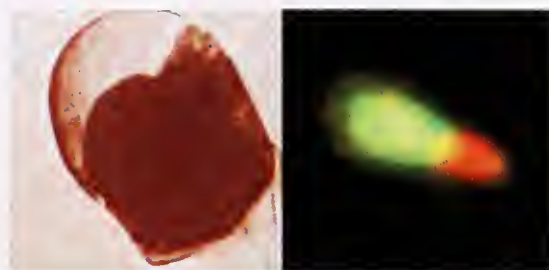


Figure 1. Left panel, trochophore larvae produced from thawed sperm. Right panel, thawed sperm stained with SYBR 14, propidium iodide and acridine orange.

Also, protocols to cryopreserve abalone sperm were designed. Acute toxic effect of cryoprotectants was determined by suspending samples of fresh abalone sperm in dimethyl sulfoxide (DMSO), propylene glycol (PG) or glycerol (Gly) at concentrations of 5%, 10%, 15%, and 20% for 5, 10, 15, 20, 25, and 30 min. The highest motility was observed in samples suspended for 5 min in 5% DMSO ($88 \pm 0.58\%$). However, no significant differences were found for sperm suspended in 5% and 10% DMSO or Gly and incubated for 5 and 10 min. Cryopreservation of sperm was held in three different freezing chambers: (1) Styrofoam box; (2) programmable rate freezing chamber (for clinical use); and (3) commercial freezing chamber (for bull sperm). The highest fertilization rate was found in samples frozen with 10% Gly in the commercial freezing chamber ($29 \pm 10\%$) (Fig 19). In addition, studies that could help to determine morphologic and ultrastructural changes produced by cryopreservation damage were performed. Membrane integrity after thawing was evaluated with the help of fluorescent dyes. The dyes SYBR 14 (100 nM) and propidium iodide (24 μ M) were used to assess membrane damage and acridine orange (38 nM) to evaluate acrosome damage (Fig 16). Also, The comet assay was used to determine DNA fragmentation. Additionally, larvae produced from thawed sperm were screened to determine chromosome abnormalities.

EFFECTS OF REMOVAL OF PUERULI FOR AQUACULTURE ON WILD CAPTURE SPINY LOBSTER FISHERIES. Bruce Phillips and Roy Melville Smith, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 68445. E-mail: b.phillips@curtin.edu.au

What affect would the harvesting of pueruli have on the wild capture fishery for the western rock lobster, *Panulirus cygnus*? The managers and industry stakeholders in the western rock lobster

fishery are committed to maintaining "biological neutrality" harvesting limits, where biological neutrality is the level of catch that would need to be forgone to maintain the reproductive capacity of the breeding stock, if pueruli are removed.

Studies have shown that natural mortality of pueruli is high. Only few western rock lobsters survive from the time of settlement as pueruli (1 year old) until they recruit into the fishery, between 3 and 5 years later. Because of this high natural mortality, the impact of possible pueruli removals for aquaculture on subsequent catches is estimated on average to be minimal (Table 3), except in the case of removal of very large quantities of pueruli, and it is not the expectation that such large numbers will ever be removed.

TABLE 3.

Estimates of the number of lobsters remaining after harvesting different quantities of pueruli in the northern region of the fishery.

Number of pueruli at settlement (millions) in an average year	Number of lobsters at the beginning of fishing (millions) without pueruli removals	Removal of pueruli (millions) for aquaculture	Number of lobsters at the beginning of fishing (millions) for different numbers of pueruli removed
600	9.55	5	9.53
		10	9.52
		20	9.49
		40	9.43

It would be possible to counter these small losses to the wild fishery, by effort reductions. For example, if it were decided to harvest 20 million pueruli from a 55 km stretch of coast in a high density settlement area, in an average puerulus settlement year, then approximately 1% of the pots in the pueruli harvesting area would need to be removed to maintain the size of the breeding stock. This converts to about 23,000 pot lifts or about 100 pots (an approximately 22 t reduction in catch) for the season.

WESTERN ROCK LOBSTER: POTENTIAL FOR ENHANCEMENT AND AQUACULTURE. Bruce Phillips, Roy Melville Smith, and Greg B. Maguire, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 68445. E-mail: b.phillips@curtin.edu.au

The Fisheries Research and Development Corporation of Australia through the Rock Lobster Enhancement and Aquaculture Subprogram has funded three projects exploring the potential for enhancing the western rock lobster wild fishery catch and developing an aquaculture industry. The first project dealt with (1) the development of techniques to harvest large quantities of pueruli and (2) ensuring that any harvesting of pueruli was biologically neutral in terms of its effect on the wild capture fishery. The key research outcome of this project was that removing pueruli will

have a very minimal effect on catches in the wild fishery—and if necessary these effects can be compensated for. Findings from this project have led to two subsequent projects, which are now in progress. One has utilized the finding of high mortality rates in the first year after settlement, to explore the possibilities for enhancing catches in the wild fishery. This may be possible by improving the survival of pueruli and early post-pueruli by designing appropriate shelters for these juvenile animals. Another will be assessing the potential for ongrowing lobsters by evaluating the growth rates and survival of pueruli to market sized lobsters, with and without refuges and at different densities. The biological data from this latter project will assist Industry investors in assessing the economic potential for western rock lobster aquaculture. Because Western Australia's immediate opportunity for rock lobster aquaculture is ongrowing of pueruli, the longer-term prospect of completing the life cycle and producing pueruli from eggs has also progressed over the last 4 years.

EFFECTS OF REEF COMPLEXITY ON HABITAT FUNCTION FOR INTERTIDAL OYSTERS. Martin H. Posey, Troy D. Alphin, Heather D. Harwell, and Thomas Molesky, Center for Marine Science, UNC-Wilmington, 5600 Marvin K. Moss Lane, Wilmington, NC 28409, USA. E-mail: poseym@uncw.edu

Oyster reefs are increasingly recognized for their habitat and ecosystem functions. Oyster reefs provide forage and refuge habitat for fauna, affect local nutrient dynamics, impact water column particulates, and indirectly influence adjacent sandflat habitats. With the decline of oyster reefs along the Atlantic and Gulf coasts, efforts are increasing to conserve existing oyster areas and to restore lost oyster habitat. However, these efforts have often been based on a presence/absence approach without consideration of landscape aspects that may influence oyster health and ecosystem function. Location of oyster reefs relative to other habitats, size of reefs, oyster density, fragmentation, and reef architecture may all influence faunal use and other ecosystem functions. For intertidal reefs, vertical complexity and edge convolution are important landscape aspects influencing habitat quality. Vertical complexity refers to the 3-dimensional roughness of the reef, with greater complexity providing more diverse spatial structure and refugia. Because the reef edge is the point of initial contact for water flow and nekton on a flood tide, varying edge characteristics may affect flow around the reef and nekton use of boundary areas. Over the past 2 years, we have examined the influence of edge complexity, vertical complexity and reef fragmentation on faunal use of intertidal reefs, oyster recruitment, growth and survival, and the influence of these parameters on the adjacent sandflat community.

Interactive and main effects of vertical complexity, edge convolution and reef fragmentation were examined using created reefs providing replicate sets of each treatment (Fig. 1) as well as sampling of natural reefs with varying surface complexity and fragmentation.



Figure 1. Artificial reef treatments: A: low surface and low edge complexity, B: low surface complexity and high edge complexity, C: high surface complexity and low edge complexity, D: high surface and high edge complexity.

Results to date indicate that the various biotic components respond differentially to aspects of reef architecture. Epifauna, especially xanthid crabs, are more abundant on high surface complexity reefs. Oyster recruitment and initial survival is greatest on low surface complexity reefs, possibly representing an indirect influence of xanthid distributions. Nekton respond more strongly to reef fragmentation than either surface or edge complexity, with greater diversity and abundances of certain taxa on fragmented reef complexes. In contrast, connections between reefs and adjacent sandflat habitats, including influences on organic content, porewater N, sediment characteristics, and microalgal biomass, are affected more by reef edge characteristics than vertical complexity. These results indicate the potential importance of landscape factors for reef habitat function and indicate the necessity of considering reef characteristics in restoration and conservation efforts.

STEROID INDUCED ENHANCED OVARIAN MATURATION IN THE GULF COAST OYSTER *CRASSOSTREA VIRGINICA*. Roberto Quintana, John Supan, and John Lynn, Louisiana State University, Baton Rouge, LA 70803, USA. E-mail: rquint1@lsu.edu

Oogenesis is a complex sequence of events endowing the ova with the cell machinery and nutrients (vitellogenins) necessary to respond to spawning cues and sperm interaction. Although invertebrate animals are not as well documented, there are emerging patterns of peptide and steroid hormones in reproductive cycles, which are similar to those in vertebrate animals. Experiments to assay the response of *C. virginica* to low level doses of estradiol-17 β (E_{17}) were performed in August and September 2001, the end of the normal maturation and spawning season of this species on the Gulf coast. Treatments were calculated based on the earlier reports of E_{17} concentrations in *C. Gigas* and calculated for body tissue mass of the whole animal estimated from shell length. Experiments ran for 14 days with a single E_{17} dose injected into the abductor muscle. Evaluation of E_{17} treatment was based on visual assessment of gonadal condition, the response of the excised whole animal to a solution of 0.01 mM 5-hydroxytryptamine (5-HT)

during a 45 min treatment period (Table 1), and histologic examination of samples taken at the gill-palp junction post 5-HT.

More females released oocytes in response to 5-HT following E_{17} treatments than the controls ($P < 0.001$, pooled data) (Table 2).

TABLE 1.

Experiment date	Dosage ng/gm wet weight	5-HT response # (%)	Total animals
8/18/01 controls		2 (20%)	8
8/18/01 estradiol		8 (58%)	14
9/15/01 controls		6 (35%)	17
9/15/01 estradiol		8 (40%)	20
9/22/01 controls		2 (13%)	30
9/22/01 estradiol		11 (35%)	32

Qualitatively, the development of gonadal tissues in treated animals was visually enhanced compared to the controls. Animals receiving lower dosages were less developed, but still notably better than controls. Histologic sections post 5-HT treatment revealed gonadal acini of the estradiol treated animals contained significant numbers of free oocytes and correlated with the animals that showed the greatest numbers of spawned oocytes. Germinal vesicles with distinct nucleol were present in all oocytes.

TABLE 2.

Estradiol treatment	Prominent genital canals # (%)	No genital canals # (%)	Total animals
controls (0 ng/g)	3 (6%)	48 (94%)	51
group 1 (37.5 ng/g)	11 (22%)	38 (78%)	49
group 2 (75 ng/g)	18 (34%)	35 (66%)	53

Control animals contained acini with necrotic oocytes even in animals that responded to 5-HT. In other controls, the ovarian mass was clearly degenerating, had few oocytes, and most of the germinal vesicles lacked a nucleolus and were vesiculated.

A series of oysters were treated with E_{17} , held for 14 days during the peak spawning period (May 2002), and examined for the presence of prominent genital canals (PGC). Treated animals showed significantly ($P < 0.001$) more individuals with prominent genital canals than control animals held under the same conditions and responded in a dose-response relation. In addition, of 265 triploid animals injected with E_{17} , 13 confirmed 3N females had developing oocytes compared to 0 of 160 untreated cohorts examined 1 year later. The overall response suggests that expansion of the methodology to determine better dose response curves, dose frequency, and response of animals will provide techniques that

are directly applicable to and useable by oyster hatchery industries for manipulating diploid and triploid broodstocks.

DISCOVERY OF GENE MARKERS THAT CAN BE USED TO RAISE THE PROFITABILITY OF ABALONE AQUACULTURE IN AUSTRALIA. Nick Robinson, Matt Baranski, Meaghan Rourke, and Ben Hayes.

Aquaculture is rapidly developing as a source of abalone for export in Australia. The aim of this study is to discover markers for genes of economic importance to the abalone industry in Australia. The most important traits to abalone farmers are rate of growth to market size, meat to shell ratio, and uniform size. Other traits of importance include flesh color, tiger patterning, texture, live product, shell shape, and frill dimensions. One hundred and fifty new microsatellite tests have been developed as part of this project. In order to maximize the power of this project to find markers affecting profitability, DNA from six full-sibling groups will be selectively pooled based on a "profit index" in which traits are weighted according to their \$ impact. Because growth rate will be a major influence in the index, and because the experimental animals have not yet grown to market size, pools of tentacle DNA from the fastest and slowest growing animals as at March 2003 were created and used to begin the experiment. Here we report on the sensitivity of the selective DNA pooling technique and on the differences that were detected between the pooled genotype profiles for fast and slow growing abalone.

TRENDS IN PINTO ABALONE *HALIOTIS KAMTSCHATKANA* ABUNDANCE AT TEN SITES IN THE SAN JUAN ISLANDS AND MANAGEMENT OF THE SPECIES IN WASHINGTON STATE. Don P. Rothaus, Robert E. Sizemore, Tina Rohela, Michael J. Ulrich, and Carolyn S. Friedman. School of Aquatic and Fishery Sciences, University of Washington, 1122 NE Boat Street, Seattle, WA 98195, USA. E-mail: carolynf@u.washington.edu

As a result of concerns regarding the stability of pinto abalone (*Haliotis kamtschatkana*) populations in Washington and the closure of the abalone fishery in neighboring British Columbia, Canada, the Washington Department of Fish and Wildlife (WDFW) established index stations at 10 sites in the San Juan Islands. These stations varied in size from 50 m² to 380 m², averaging about 220 m². WDFW divers systematically surveyed each of these stations in 1992, 1994, and 1996.

A decrease in total abalone abundance at these 10 index stations from 1992 to 1994 ($n = 351$ to $n = 288$), along with anecdotal information of population decline by University of Washington (UW) researchers and WDFW Enforcement personnel, resulted in the closure of the Washington pinto abalone fishery in 1994. Following the closure, a 2003 survey documented a declined from 351 in 1992 to 137 in 2003 and densities below or near

0.1/m². Research in other regions indicate that sedentary invertebrates, such as abalone, must be within 1.0–2.0 m of one another ($d \geq 0.33$ –0.15 abalone/m²) for successful fertilization. However, given the gregarious nature of abalone, we examined the proximity of individuals to one another and observed that most animals were actually within 1–2 m of one another. At one site, of the 18 abalone observed and an average density of 0.1 per m², when the proximity of individuals to one another was measured, 44% were within 0.3 m of another abalone, 72% were within 1 m, 83% were within 2 m, 94% were within 3 m, and 100% were within 4 m of another individual within the index station. Based on survey data, and information from abalone fisheries around the world, it is clear that additional stock assessments are needed to analyze population trends and abalone behavior in Washington abalone stocks to best manage this species. Additional index sites, early juvenile life history, population genetics, and the potential for enhancement are also being examined in this study.

SUMMER MORTALITY IN THE PACIFIC OYSTER *CRASSOSTREA GIGAS*, OVERVIEW OF 3-YEAR RESULTS OF THE COOPERATIVE "MOREST" PROJECT. J. F. Samain, P. Boudry, L. Degremont, P. Soletchnik, M. Ropert, E. Bedier, J. L. Martin, J. Moal, M. Mathieu, S. Pouvreau, C. Lambert, J. M. Escoubas, J. L. Nicolas, F. Le Roux, T. Renault, T. Burgeot, and C. Bacher, Laboratoire IFREMER de Physiologie des Invertébrés, Centre de Brest, BP 70, 29280 Plouzané, France. E-mail: jfsamain@ifremer.fr

Summer mortality of *C. gigas* oyster is not yet clearly understood in literature. Complex interactions are suggested between environment, oysters, and pathogens. Such multifactorial event led us to organize a research network (including genetics, physiology, immunology, pathology, ecotoxicology, and environment) collaborating on the same natural or hatchery biological material. This strategy allowed to progressively classify importance of the different factors involved in the phenomena by coupling field and experimental studies.

A temperature over 19 °C is the first condition in France. This temperature is associated to *C. gigas* reproductive period. Defense responses are lowered during this reproductive stage contrary to sterile triploids that are mainly resistant, corroborating that reproduction is also a risky biological stage. A genetic component evidenced by divergent selection in two generations was confirmed the third year (Boudry et al. this meeting). Sensitive (S) and resistant (R) oysters in the same area or in controlled experimental conditions showed a difference in reproductive strategy faced to similar food conditions (Fig. 1).

High trophic conditions increased the oyster reproductive effort and so the risky phase as shown by a negative scope for growth and a higher sensitivity to bacterial challenges. However, some stresses were necessary to induce mortality when temperature and reproduction were in the risky window. A simple transfer of oyster

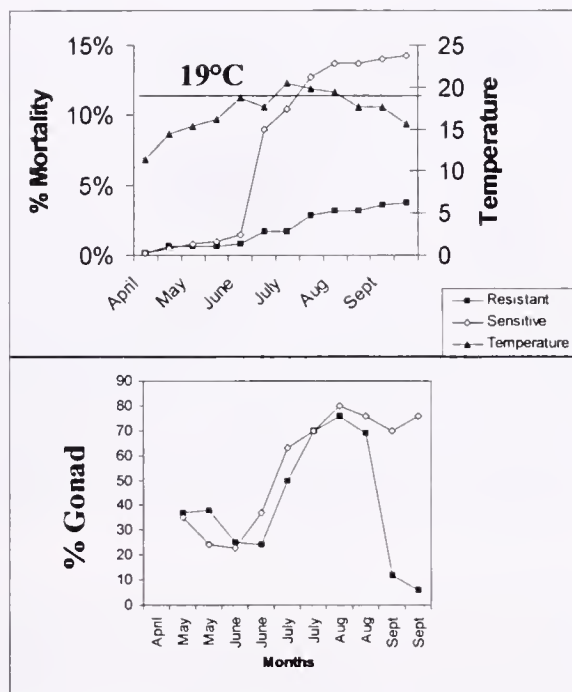


Figure 1. Up: cumulative mortality (%) of R and S oysters in the field (South Brittany) and water temperature (°C). Down: corresponding % gonad in the flesh (down).

induced mortality in these conditions. Sediment proximity appeared also as a detrimental factor, because oysters at 10 cm from sediment died contrary to oysters at 70 cm.

Different strains and species of vibrios were isolated from moribund oysters and determined by molecular techniques. Their virulence tested by injection in the muscle appeared variable according to strains and species. Effect of environmental factors on their virulence expression is also one among the key points under study.

USING PEARL OYSTERS AS HEAVY METAL MONITORS IN TROPICAL WATERS. Dale J. Sarver, Aaron Ellis, Neil Anthony Sims, and David Wise, Black Pearls, Inc., P.O. Box 525, Holualoa, HI 96725. E-mail: www.blackpearlsinc.com

Accurate and cost-effective methods are needed for monitoring heavy metal pollution levels in tropical oceans. Direct measurement of metals in seawater is inappropriate for most tropical ocean areas, as it requires regular sampling programs, expensive equipment, and sophisticated analytical expertise. Further, metal pollutants are usually sequestered in sediments, and water samples often miss the major peaks of exposure when sediments are resuspended during storms or other events. Because of these inadequacies, marine filter-feeding bivalves, such as mussels (*Mytilus edulis*), are widely used as bioaccumulators for marine pollution monitoring. The "Mussel Watch" system has proven very successful, and con-

tinues to be the most comprehensive measure of coastal marine metal pollution in the United States. However, these mussel and oyster species are limited to temperate water regions of the globe, and there are no comparable subjects for monitoring of oligotrophic tropical waters.

Our research results demonstrate that pearl oysters are the ideal warm-water complement to the "Mussel Watch" work. *Pinctada* spp are cosmopolitan in distribution, sessile, and long-lived. Initial trials have confirmed a phenomenal propensity to accumulate heavy metals in the Hawaiian pearl oyster (*P. margaritifera* *galtsoffi*). In controlled tank trials, copper, cadmium, and zinc showed constant rates of bioaccumulation in pearl oyster tissues, directly proportional to the metals' concentration in the tank water, and the duration of exposure. These trials also established standards for field monitoring, with demonstration trials providing preliminary data from around the Hawaiian Islands. A second series of field trials underscored the significant temporal variability that typifies heavy metal monitoring.

Ongoing research is now expanding the range of metals to include strontium, cobalt, and lead. Monitoring levels of radioactive strontium and cobalt could be an invaluable tool in remediation and repopulation of atolls in the South Pacific (such as Bikini and Enewetak Atolls, in the Marshall Islands, Christmas Island, in Kiribati, or Muroroa, in French Polynesia), which were sites of earlier atmospheric and underground nuclear bomb tests by the United States, Britain, and France.

USING OYSTER REEF COMMUNITIES IN THE DESIGN OF ESTUARINE RESTORATION PROJECTS IN SOUTH FLORIDA. Michael Savarese, S. Gregory Tolley, and Aswani K. Volety, Florida Gulf Coast University, 10501 FGCU Blvd., Fort Myers, FL 33965, USA. E-mail: Msavares@fgcu.edu

Greater Everglades restoration in South Florida is the largest public works project in US history. Although most associate the Everglades with freshwater wetlands, restoration projects concern both terrestrial and estuarine habitats and focus on entire watersheds. The ultimate goal of Everglades restoration is to "get the water right"—to re-establish the pre-alteration quantity, quality, timing, and distribution of freshwater. The process is governed by a federally legislated partnership between the United States Army Corps of Engineers and the South Florida Water Management District and operates through consensus among numerous governmental agencies, NGOs, and university professionals. Restoration planning follows a strict protocol that includes both scientifically and managerially sound practices. As the downstream recipient of freshwater, estuaries have much to gain from watershed restoration. The American oyster, *Crassostrea virginica*, is being used as a bioindicator of estuarine health, as a tool for establishing restoration targets, and as a measure of restoration effectiveness. The purpose of our presentation is three-fold. First, the restoration protocol used in the Greater Everglades is reviewed. Second, the

usefulness of oyster reef biology and ecology to this process is demonstrated. Lastly, we illustrate this application with an example from an estuarine restoration project that is designed and awaiting authorization.

The protocol adopted for Everglades restoration consists of 9 steps: (1) defining restoration goals; (2) characterizing the current conditions; (3) establishing the pre-alteration state; (4) designing alternative restoration scenarios; (5) establishing performance measures and targets; (6) modeling to evaluate each scenario; (7) designing a restoration effectiveness monitoring plan; (8) implementing a restoration scenario; and (9) initiating adaptive management. Oysters and their reef communities are being used in steps 2, 3, 5, and 7. Various aspects of oyster physiology and ecology, including growth, standing stock, recruitment, susceptibility to disease, living density, the distribution and aerial extent of reefs, and the diversity and richness of the reef community, serve as bioindicators of estuarine health (step 2). These aspects are compared using a spatial homologue approach, whereby geomorphologically similar positions along the estuarine axis are compared among altered and pristine estuaries. Step 3 is achieved by comparing the present distribution of oyster reefs with pre-alteration, historical surveys. In the absence of such documents, the stable isotope and trace metal geochemistry of subfossil oyster shells can be used to infer paleosalinity. The same aspects of physiology and ecology are used to define targets and performance measures of the restoration project (step 5). Finally the restoration project's success can be gauged (step 7) by how close the system approaches a given target.

FIRST HARVEST OF BLACK PEARLS FROM THE IMPERILED NATIVE HAWAIIAN PEARL OYSTER *PINCTADA MARGARITIFERA GALTISOFFI*. Neil Anthony Sims and Dale J. Sarver, Black Pearls, Inc., P.O. Box 525, Holualoa, HI 96725, USA. E-mail: www.blackpearlsinc.com

The native Hawaiian black-lip pearl oyster, *Pinctada margaritifera galtsoffi*, is a distinct, endemic subspecies of the fabled Tahitian pearl oyster, *P. margaritifera*. The Hawaiian pearl oyster was once common, and was traditionally used by Hawaiians in making fishing hooks and lures, other tools, and ornaments. The oyster has become increasingly rare since Western contact, due primarily to commercial fishing. The last significant stocks of *P. margaritifera galtsoffi* were fished out at Pearl and Hermes Reef in the 1920s, when over 100 tons of pearl shell were taken from this shallow, open lagoon. Recent surveys by NMFS divers found only 30 adult shells in over 18 diver-hours. All of these oysters were above 20 cm shell diameter, suggesting that recruitment is negligible. Stocks remain uncommon around the main Hawaiian islands, despite legal protection. Relict stocks in a few areas, such as Kaneohe Bay, still show decline.

Although hatchery techniques are now well-established for this species, stock recovery in Hawaii is limited by heavy predation on

the reef, pilfering by divers, pollution in protected reef and lagoon areas, and low water-residence time for larvae in open reef systems. Predation by fish and octopi is very heavy. Grow-out trials in protective cages left oysters vulnerable to *Cymatium* and other predatory snails settling out of the plankton. The most effective stock re-establishment plan would therefore be to set up reproductive reserves of large, densely-aggregated, older adult oysters. A "reproductive node" of closely-packed, highly fecund animals would be able to synchronize spawning and achieve high fertilization rates, resulting in large numbers of larvae. These larvae would then be dispersed by currents, eventually settling out naturally onto the reefs and lagoons throughout the group. Serendipitously (or otherwise), such a "reproductive node" can be provided almost precisely by a commercial pearl farm operation.

Black Pearls, Inc. (BPI) has therefore been developing the concept of Hawaii's first pearl farm as both a commercial business and a conservation tool. In essence, the pearl farm becomes a financially-self-sustaining (or profitable) means for resource enhancement. BPI assisted in the rewriting of Hawaii's ocean leasing legislation, and has since completed the lease application process for a 75-acre area next to Honolulu's International Airport. In 2003, BPI harvested the first genuine "Hawaiian pearls" from this site. This now provides a distinctive line of local pearls and pearl shell jewelry, an opportunity for Native Hawaiian artisans to once again work with their local material, and an added romantic allure to the islands.

MANAGING *PERKINSUS MARINUS* IN THE GULF OF MEXICO: THE DERMOWATCH PROGRAM. Thomas M. Soniat, Mason Foret, Department of Biology, Nicholls State University, Thibodaux, LA 70310; Sammy M. Ray, Department of Marine Biology, Texas A&M University at Galveston, Galveston, TX 77553; Enrique V. Kortright, Kortright Corporation, 102 Allendale Dr., Thibodaux, LA 70301; Lance Robinson, Texas Parks & Wildlife Department, 1018 Todville Rd., Seabrook, TX 77586, USA.

Throughout its range from Mexico to Maine, *Perkinsus marinus* is a major cause of mortality in eastern oysters, *Crassostrea virginica*. The parasite was first described from Louisiana oysters as *Dermocystidium marinum*, from which its common name "Dermo" is derived. Because there is no economical or feasible treatment of infected oysters, the only effective approach to control Dermo is proper management. The parasite is more prevalent at high water temperature (T) and high salinity (S) and is thus most problematic during late summer and on the seaward side of estuaries. Possible management techniques include maintaining freshwater inflows, diverting fresh water into high-salinity estuaries, early harvest of infected oysters, and moving infected oysters to low salinity waters. (The movement of infected oysters to low salinity environments is not recommended in estuaries lacking

frequent freshets, because this practice may lead to the long-term establishment of the parasite in the upper estuary).

The DermoWatch Program was established to more effectively manage Dermo in the Gulf of Mexico. DermoWatch is a web site (www.blueblee.com/dermo), a monitoring program, and an online community for the management of *P. marinus*. The web site contains an embedded model that calculates a time to critical level (t-crit) of disease from an initial level of disease and water T and S. Thus, samples of oysters are collected and water T and S are measured. An initial weighted incidence (i-WI) of parasitism is determined using Ray's fluid thioglycollate method. A critical WI (c-WI) is set at 1.5. Water T, S, i-WI are inputs to the model and by simulation a t-crit is determined as the number of days to reach c-WI, assuming no change in T or S.

Six public reefs and three private leases in Galveston Bay have been sampled monthly since December 1998. New Texas sites and Louisiana sites are being added. The web site displays the most recent data from each site on the home page and archives all data, such that an historical record is maintained. A web-accessible "Dermo Calculator" provides anyone with data on T, S, and i-WI the opportunity to calculate a t-crit and explore the dynamics of disease progression.

An obvious limitation of the approach is that single values of T and S, taken at the time the oysters are sampled, are used to represent environmental conditions for the entire month. We are testing the utility of a continuous monitoring station to better predict disease progression (<http://weather.lumcon.edu/stationdata.asp?stationid=105>). Studies are also being conducted on the relationship of levels of disease (WI) to oyster mortality. A clearer understanding of the relationship between WI and mortality will help us to more precisely establish a c-WI.

NUCLEUS QUALITY AND SUBSTITUTE OF ROUTINE SHELL. Ajai K. Sonkar, Pearl Aquaculture Research Foundation, 557/470, Old Katra Allahabad 211 002, India.

A study was undertaken to answer several questions for the particularity of the shell for the nuclei of pearls such as properties of a suitable nucleus, what makes nucleus perfect for seeding in an oyster...its hardness? Its density? Its surface smoothness? Its color? Or its brightness?

Why is freshwater shell only suitable for nuclei, does it affect the pearl quality? Or is it suitable because it is easy to drill? Does shell of the nuclei decide the quality of pearl or acceptability by the recipient oyster? With a nucleus cut from saline water shell can equal ability to be drilled and perfect surface smoothness and similar in all above factors produce a similar quality of pearl?

The author tested several shells from both saline and freshwater to find a substitute for the routinely used freshwater shell and received interesting results. All above aspects of the nucleus and the results of the experiments/comparison tests are discussed in the paper.



Figure 1. Nuclei cut from other than routine shells.

A COMPARISON OF RECRUITMENT ON MID-WATER OYSTER SPAT COLLECTORS AND SUBSEQUENT BOTTOM RECRUITMENT IN THREE SUB ESTUARIES OF THE CHESAPEAKE BAY, VIRGINIA. M. Southworth and R. Mann, School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA.

Shellstrings have been used as oyster spat collectors for over 40 years to estimate potential bottom recruitment of oysters in many of Virginia's sub estuaries. Until recently quantitative estimators of subsequent bottom recruitment have been lacking. We offer a comparison of estimates of cumulative spat recruitment obtained from shellstrings throughout the settlement period (recorded on a weekly basis as the number of spat per shell and then totaled over the course of the settlement period) and quantitative estimates of spat settlement obtained from patent tong sampling in the subsequent fall. Comparisons include co-located stations collected over a 5-year period in the James River (8 stations), Piankatank River (7 stations), and the Great Wicomico River (6 stations) from 1998 to 2002. We examine the following aspects of the spat collector to benthic recruitment relationship: all data irrespective of source and year, location specific relationships (within and between rivers), the temporal effects of settlement on subsequent recruitment, and absolute levels of settlement on subsequent recruitment.

RECOMMENDATIONS FOR CULTURING JUVENILE QUEEN CONCH *STROMBUS GIGAS* FOR RESTOCKING AND COMMERCIAL PURPOSES. Ashley Spring and Megan Davis, Florida Institute of Technology, Biology Department, 150 West University Blvd., Melbourne, FL 32901, USA. E-mail: aspring@hboi.edu

Queen conch, *Strombus gigas*, are overfished throughout Florida and the Caribbean. In an attempt to curtail the depleted wild stocks, queen conch aquaculture has developed through the last 40 years. Whether the goal is to culture queen conch for stock enhancement, the seafood market, or the aquarium trade, there remain a number of fundamental aquaculture methods to improve

upon to enhance the quality of cultured conch and reduce costs. This study aims to improve survival, growth, shell strength, and culture costs of juvenile queen conch by manipulating stocking density and tank substrate. Three experiments were designed to test the effects of five stocking densities (20, 50, 75, 200, and 400 conch/m²) and three tank substrates (crushed coral aragonite, plastic liner, and aragonite chips) in recirculating water systems over 16 months.

Conch grown at low densities grew significantly faster (0.11–0.13 mm/day) than conch grown at high densities (0.06–0.08 mm/day). On the other hand, conch grown at high densities possessed significantly stronger shells. High mortalities occurred in conch grown on plastic liner (60%) versus aragonite chips (97%) and crushed coral aragonite (91%). There were no differences in growth between conch grown on the two sand substrates. Crushed coral aragonite costs one-third of the price of aragonite chips and is therefore suggested as a less expensive substrate option.

To culture juvenile queen conch for stock enhancement purposes, it is recommended that conch are grown at a stocking density of 200–400 conch/m² to provide the animals with the strongest shell strengths and on aragonite chips, which may increase burial rates. Commercial markets are not concerned with shell morphology; therefore, the fastest and least expensive culture conditions are recommended. Conch grown for the seafood market and the aquarium trade will grow the fastest at stocking densities of 20–50 conch/m² and can be grown inexpensively on crushed coral aragonite. The recommended culture conditions may benefit aquaculture facilities, fishery managers, and commercial industries with higher quality stocks at lower costs.

INDUCED TRIPLOIDY IN THE BAY SCALLOP *ARGOPECTEN IRRADIANS* AND EARLY FIELD CULTURE PERFORMANCE. Amandine S. Surier* and Richard C. Karney, Martha's Vineyard Shellfish Group, PO Box 1552, Oak Bluffs, MA 02557, USA. E-mail: mvsg@capecod.net

Triploidy is the condition of possessing three times the haploid number of chromosomes in the cell nucleus. This condition can be induced by cell treatment, in the early stages of development. Not to be confused with controversial, transgenic genetically-modified organisms (GMO's), polyploidy is simply an increase in the number of a single organism's chromosomes, with no introduction of foreign genetic material. Triploid animals and plants are usually sterile because of the inability of homologous chromosomes to synapse in meiosis. As a result of their sterility, they tend to divert the energy usually used for reproduction towards somatic growth. Because of this unique quality, triploid bivalve production has attracted worldwide attention since the early 1980's. At present triploidy has been successfully applied to the economic benefit of the Pacific oyster industry on the west coast of the US and also in France.

Triploidy in scallops has been investigated in several species over the past 20 years. Overall, research suggests that the development of a triploid bay scallop will result in an increased growth rate, a larger adductor muscle mass and an increased hardness with regard to stressful environmental conditions.

To test this hypothesis, triploidy was induced in the bay scallop *Argopecten irradians* under funding from the Northeastern Regional Aquaculture Center. The newly fertilized eggs were treated with a low risk chemical, 6-DMAP, that has been shown to be slightly less efficient than Cytochalasin B but much safer to handle as well as being water soluble. The success of induction was measured by flow cytometry at the Virginia Institute of Marine Science. Triploidy was induced on five occasions during the 2003 culture season. Each batch of eggs was treated 15 to 25 minutes post fertilization for approximately 11 minutes with a treatment concentration of 400 μ M. The results ranged from 77% up to 100% triploidy in the first week of development. In late August, early September, juveniles from the last two spawns were deployed in growth trials in Katama Bay on Martha's Vineyard, MA, Buzzards Bay on Cape Cod, MA, in Point Judith Pond, RI, and in Cedar Beach on eastern Long Island, NY. Shell growth was measured every other week at each site until the end of the growing season. There was no significant difference in juvenile shell growth between triploids and diploids at any of the sites. The animals were overwintered in pearl nets. The survivors will be deployed in the spring for further growth monitoring, and adductor muscle weight and gonad development analyses.

REFOCUSING THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*) STOCK ENHANCEMENT PROGRAM. M. Tlusty, New England Aquarium, Central Wharf, Boston MA 02110, USA. E-mail: mtlusty@neaq.org

The American lobster (*Homarus americanus*) stock enhancement program is currently non-existent. Although stock managers have been able to culture this species in captivity for over a century, attempts to enhance wild stocks have largely failed. This is in direct opposition to the European lobster (*H. gammarus*), which can and is being successfully enhanced. The best example for this species is in Norway where 50% of the fishery landings are of animals that began life in a hatchery.

The respective success and failure of the European and American strategies was directly influenced by the respective management strategies. The strategy of the American lobster stock enhancement program was to rear a large number of fourth stage, early benthic juveniles. In contrast, European programs rear fewer animals but for a longer period. Animals are reared for up to a year, to the ninth or twelfth stage. The biologic basis for the success and failure of the respective programs will be discussed.

To refocus the American lobster stock-enhancement program, the management philosophy needs to be shifted to rear animals to later stages. Part of the reluctance in making this shift was the

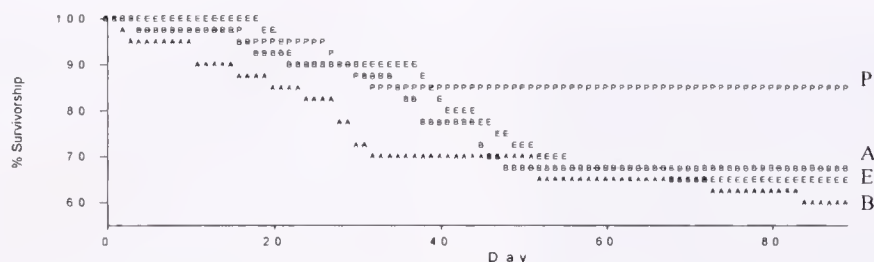


Figure 1. The survivorship of juveniles (beginning with stage IV animals) fed one of four different diets. Diets are frozen *Artemia* (B), or the commercial *Artemia* replacement (CAR) diets Artemac (A), Economac (E), or Progression (P).

higher cost of the longer hatchery phase. Thus, refocusing will entail creating a cost effective rearing environment, which needs to be demonstrated. At the New England Aquarium, research is being conducted on creating a cost effective rearing environment. A rearing method was developed that can grow lobsters individually to ninth stage at a density of 400/m², compared to approximately 150/m² for European lobsters in communal culture, and 10/m² in the wild. In addition, the use of commercial *Artemia* replacement diets is being investigated for their role in decreasing the cost of producing older animals (Fig. 1). Demonstrating the ease of individual rearing, the availability of commercially available formulated feeds, and a decreased cost of production are the first steps necessary in refocusing the American lobster stock enhancement program.

These are the first steps necessary in refocusing the American lobster stock enhancement program. Future efforts will include examining the performance of hatchery animals reared on commercial *Artemia* replacement diets when released into the wild, and determine full-scale economic production scenarios. Based on the recent advanced in decreasing the cost of producing later staged juveniles, the future for American lobster stock enhancement is very promising.

ASSESSING ENVIRONMENTAL IMPACTS OF AMERICAN LOBSTERS IMPOUNDMENTS IN COASTAL MAINE, USA. Michael Tlusty, New England Aquarium, Central Wharf Boston, MA 02110, USA. E-mail: mtlusty@neaq.org

In the American lobster fishery, animals caught in the fall are held live in dammed tidal areas known as impoundments. They are a critical part of the fishery, as they prevent a market glut in the fall, enabling constant supplies of animals through the winter. In the United States, Maine has 65 impoundments, with a combined capacity of 2,600 metric tons. While impounded, lobsters are fed leading to a substantial input of organic matter. As with other aquaculture operations, this can potentially impact the environment through increased sedimentation rates, and the creation of anoxic sediments. Although these impoundments have been operating for more than a century, there are no requirements for envi-

ronmental monitoring, and have been no studies of the impacts of these areas.

In 1999 and 2000, nine lobster pounds and reference areas were monitored in southern Maine to determine the flow of organic matter through these areas. Macrofauna and microfauna were also monitored to assess subsequent environmental effects. Impoundments had little to no measurable impact on the accumulation of organic matter. Lobster feed was nearly 90% organic matter (loss on ignition at 500 °C), whereas feces ranged from 20%–40% depending on feeding frequency and temperature. Benthic organic matter ranged from 1.5%–7%. There was no discernable trend if values were greater inside compared to outside the pound. Overall, the holding of American lobsters at high densities in impoundments is not resulting in a large amount of organic matter being deposited to the benthos. Possible reasons for this include rapid water recycling, frequent exposure to air, and long fallow periods every year.

MANAGING OYSTER REEFS FOR ECOLOGICAL FUNCTION: FACTORS INFLUENCING HABITAT QUALITY FOR REEF-RESIDENT COMMUNITIES. S. Gregory Tolley, Aswani K. Volety, and Mike Savarese, Florida Gulf Coast University, College of Arts and Sciences, 10501 FGCU Blvd South, Fort Myers, FL 33965, USA. E-mail: gtolley@fgcu.edu

To gauge the influence of freshwater inflow on the habitat value of oyster reefs, spatial and seasonal patterns of the presence of reef-resident fishes and decapods were assessed in the Caloosa-hatchee, Estero, and Faka-Union estuaries of Southwest Florida. Lift nets (1 m² containing 5 L of oyster clusters) were deployed on intertidal reefs at three sites along the salinity gradient of each estuary. Nets were deployed during three seasonally dry and three seasonally wet months for a period of 30 d. Oyster densities were estimated at each site and a number of community metrics were calculated as a measure of habitat value (eg. abundance, biomass, presence of oyster-dependent organisms, diversity, dominance, and richness).

Several metrics trended downstream in one or more systems (eg. abundance and biomass increased downstream in all three systems, and measures of biodiversity were higher downstream in

the Caloosahatchee) and seemed to be unrelated to the density of living oysters present. Although abundance was higher during the wet season for all three systems, biomass was lower during this season in the Caloosahatchee. In the Caloosahatchee and the Estero, measures of biodiversity were higher during the dry season. A number of metrics were positively correlated with salinity in one or more systems: biomass, biodiversity (Fig. 1), and the presence of oyster dependent species. Additional data collected in the Caloosahatchee demonstrated that some metrics were also related to freshwater inflow; for example, biomass and biodiversity varied inversely with inflow recorded during the sampling period as well as with inflow recorded at time lags of up to 3 to 4 months.

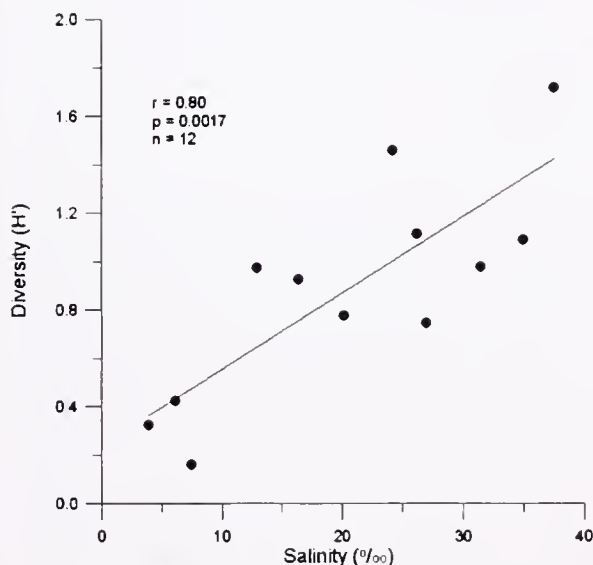


Figure 1. Relationship between diversity of reef residents and salinity of capture in the Caloosahatchee estuary.

These results suggest that regulated freshwater releases can be a useful tool for managing Southwest Florida oyster reefs, but that resource managers should consider the habitat requirements of oyster-reef organisms in addition to those of the oysters themselves when establishing release guidelines.

ROLE OF ANTHROPOGENIC AND ENVIRONMENTAL VARIABILITY ON THE PHYSIOLOGICAL AND ECOLOGICAL RESPONSES OF OYSTERS IN SOUTHWEST FLORIDA ESTUARIES. K. Aswani, S. Volety, Gregory Tolley, Michael Savarese, and James T. Winstead, Florida Gulf Coast University, 10501 FGCU Blvd., Fort Myers, FL 33965, USA. E-mail: volety@fgcu.edu

The role of freshwater alterations and seasonal changes on the ecological and physiological responses of oysters were investigated in the Caloosahatchee River, Estero Bay and Faka-Union

estuaries in SW Florida. Condition index, oyster density, and disease incidence of *Perkinsus marinus* were measured through monthly sampling at three comparable sites located along the main salinity gradient in Estero Bay and Faka-Union estuary, while spat recruitment, juvenile growth, and gonadal index of oysters were also measured monthly at five locations along the salinity gradient from the Caloosahatchee River.

Sharp declines in temperatures and salinities resulted in decreased *P. marinus* infections in oysters from all locations. Antagonistic effects of temperature and salinity on *P. marinus* infections make trends difficult to discern. Oysters from Faka-Union, an estuary that receives excessive freshwater, have a significantly lower condition index compared to a more natural estuary, Estero Bay (Fig. 1).

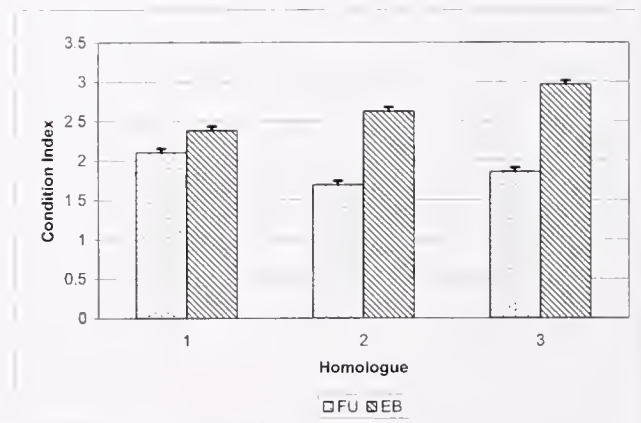


Figure 1. Condition index of oysters from the Faka-Union (FU) and Estero Bay (EB) estuaries. Stations 1, 2, and 3 are from upstream to downstream.

Condition index in oysters from all three estuaries was influenced by the reproductive activity. Oyster density was higher in the upper-mid estuary and decreased with increasing distance offshore in the Estero Bay and the Caloosahatchee River, whereas none to few oysters are present at the upstream locations in the Faka-Union estuary. Only the downstream-most site showed appreciable living densities, a pattern consistent with predicted effects of watershed alteration and freshwater inundation. Oysters in SW Florida estuaries seem to continuously spawn between March/April to October, with upstream oysters spawning earlier than the downstream oysters. Freshwater releases from Lake Okeechobee into the Caloosahatchee River in spring/summer months resulted in >95% mortality of juvenile oysters and pushed the loci of spat recruitment to more downstream locations.

We are communicating the results of our study with resource managers who are utilizing them to adjust the quantity and timing of freshwater releases into the estuaries. The use of adaptive man-

agement approaches involving freshwater releases to sustain and enhance oyster populations is valuable to the ecology of SW Florida estuaries.

LOCALIZATION OF TAACC REPEATS TO TELOMERES OF *PENAEUS VANNAMEI* CHROMOSOMES BY FISH.

Yongping Wang, Ximing Guo, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Morris, NJ 08349 USA; **Acacia Alcivar-Warren**, Environmental and Comparative Genomics Section, Department of Environmental and Population Health, Tufts University School of Veterinary Medicine, 200 Westboro Road, North Grafton, MA 01536, USA; and **Linghua Zhou, Jianhai Xiang, and Yongping Wang**, Institute of Oceanology, Experimental Marine Biology Laboratory, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, China.

Telomeres are the protein-DNA structures at ends of linear chromosomes. They protect chromosomes from degradation and end-to-end fusion, rearrangement and chromosome loss. Telomeres may play an important role in aging and oncogenesis. Telomeric DNA usually consists of tandem repeats of a very simple sequence about 5–10 bp in length, which are often highly conserved across taxa. All vertebrates studied so far, as well as some other organisms such as protozoa *Trypanosoma* and several slime molds and fungi, share the same telomere sequence (TTAGGG)_n. Telomere sequences in marine invertebrates are not extensively studied. Two groups of ciliates, *Tetrahymena* and *Oxytricha*, have different telomeric sequences of (TTGGGG)_n and (TTTTGGGG)_n respectively. In mollusks, 10 bivalves studied so far contain the vertebrate sequence, (TTAGGG)_n.

In this study, TAACC repeats were localized to telomeres of *Penaeus vannamei* chromosomes by fluorescence *in situ* hybridization (FISH). Clones containing TAACC repeats were identified by sequencing, and primers were designed to amplify DNA fragments containing TAACC repeats. Four FISH probes, P1–P4, containing different numbers of TAACC repeats were produced by PCR amplification and labeled by incorporation of digoxigenin-11-dUTP during PCR. Metaphase material was prepared from testis. The chromosome number of *P. vannamei* was confirmed as $N = 44$. All four probes produced positive FISH signals on telomeres of *P. vannamei* chromosomes. A few chromosomes had positive signals interstitially. The four probes differed considerably in signal strength and chromosome coverage. Despite some variation, signal strength and chromosome coverage of the four probes were in the general order of $P1 > P2 > P3 > P4$, which correlated nicely with the length of TAACC repeats within the four probes: 83, 66, 35, and 30 bp, respectively. The correlation between signal strength/chromosomal coverage and the length of TAACC repeats suggests that TAACC repeats, rather than flanking sequences, produced the FISH signals at chromosome ends, and TAACC is likely the telomere sequences for *P. vannamei*.

PRODUCTION AND EVALUATION OF ALL-TRIPLOID AND DISEASE-RESISTANT EASTERN OYSTERS FOR AQUACULTURE. **Yongping Wang, Ximing Guo, Gregory DeBrosse, and Susan Ford**, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA. E-mail: wang@hsrl.rutgers.edu

The eastern oyster (*Crassostrea virginica*) faces two major diseases: MSX (caused by the parasite *Haplosporidium nelsoni*) and Dermo (caused by the parasite *Perkinsus marinus*). Rutgers University has been breeding oysters for disease-resistance since the early 1960s. Strains resulting from the Rutgers breeding program have shown strong resistance to MSX and recently some resistance to Dermo. Because the Rutgers strains are superior in disease-resistance, they do not grow as fast as some of the commercial strains, such as the Flower's Oyster Company's strain (which shows little resistance to MSX and Dermo). Growth improvement of the Rutgers disease-resistant strains is needed to maximize return for oyster farmers. Fast growth means reduced culture duration/cost, and exposure to diseases. We recently developed tetraploid oysters from the Rutgers disease-resistant strains. The goal of this study is to produce natural triploids from the disease-resistant tetraploid stocks and compare their growth and survival to that of normal diploids and chemically induced triploids. Triploids grow significantly faster than diploids in most mollusks studied so far.

Three replicates of 3 groups were produced from Rutgers disease-resistant stocks: (1) diploid control (2N); (2) triploids induced by blocking polar body II with cytochalasin B (3nCB); and (3) natural triploids produced by diploid female \times tetraploid male crosses (3nDT). The 3nCB groups produced 72% triploids on average, and the 3nDT groups produced 98% triploids. Oysters from 3nCB and 3nDT groups were combined with diploid controls so that the combined groups contained approximately 50% diploids and 50% triploids from either 3nCB or 3nDT groups. The diploids served as internal controls, and growth comparison was made only after ploidy determination for each individual. No significant difference in growth and survival was detected between diploids and 3nCB or 3nDT triploids at 1 year of age. Another sampling is scheduled at 18 months, and data will be presented.

CONSERVATION AND LIFE HISTORY STRATEGIES OF ENDANGERED AND AT RISK SPECIES OF ALASMIDONTA (BIVALVIA, UNIONIDAE). **Barry J. Wicklow**, Department of Biology, Saint Anselm College, Manchester, New Hampshire 03102, USA. E-mail: bwicklow@anselm.edu

Freshwater mussels suffered astonishing declines in diversity, abundance, and distribution within the last 200 years. In the genus *Alasmidonta*, 9 of 13 species are threatened, endangered or extinct. Here I compare the complex life histories of the dwarf wedgemussel, *A. heterodon* (federally endangered), the brook floater, *A. varicosa* (state endangered throughout most of its range), and the

triangle floater, *A. undulata* (stable or special concern in the NE, threatened or endangered in the SE). New Hampshire glochidial release periods are from early March to June for *A. heterodon*, mid-April to May for *A. varicosa*, late April to June for *A. undulata*. The only *Alasmidonta* species using a behavioral display to attract host fishes is *A. heterodon* which utilizes as host species the tessellated darter, *Etheostoma blunstedti*, the slimy sculpin, *Cottus cognatus*, and the Atlantic salmon, *Salmo salar* in the NE. *A. varicosa* and *A. undulata* use a wider range of host fish species. Long-term studies show human associated population density declines of *A. varicosa*. Human impacts, including stream fragmentation, disrupt life cycles, prevent host fish migration, block gene flow, and prohibit recolonization resulting in reduced recruitment rates, decreased population densities, and increased probability of local extinction.

DEVELOPMENT OF SECURE REARING SYSTEMS FOR DOMESTICATED BIVALVE BROODSTOCK. Gary H. Wikfors, James C. Widman, Barry C. Smith, David Veilleux, Joseph Choromanski, Shannon L. Meseck, Ronald Goldberg, and Sheila Stiles, NOAA Fisheries and Ecosystems, NEFSC, 212 Rogers Avenue, Milford, CT 06460, USA.

An intensive, land-based nursery culture system using recirculating water principles is being designed and tested for post-set bivalves. One additional potential application of such a system is to secure valuable genetic resources, such as domesticated broodstock. These genetic resources could be threatened by uncontrolled environmental events such as storms, disease outbreaks, harmful algal blooms, pollutant spills, etc. Accordingly, a rearing environment for highly specialized broodstock is needed that is more secure and dependable than nature provides.

A recirculating system for bivalves faces some of the same imperatives as finfish systems, (eg, the need to maintain chemical and biological water quality) however, some challenges are unique to bivalves. First, a nutritionally complete diet must be available continuously; second, biofilters and aeration need to be effective in maintaining chemical water quality without consuming the small feed particles used by bivalves; and third, respiratory and nutritional needs of the shellfish must be balanced such that food is not filtered wastefully to maintain respiration rates. Our land-based, recirculating seawater bivalve rearing system consists of: (1) a solar microalgal culture unit, (2) a shellfish-rearing unit with several different biofilter options, and (3) a seawater discharge treatment unit using seaweed to remove dissolved nutrients from the waste stream. Although the system design and operation have not yet been optimized, experiments have been conducted to test and parameterize automated monitoring and process control protocols

for the three component units. Findings—and remaining problems—identified in our research thus far will be summarized.

THE NORTHERN QUAHOG *MERCENARIA MERCENARIA* HAS SLOWER GROWTH AND POORER CONVERSION EFFICIENCY THAN OYSTERS OR SCALLOPS FED IDENTICAL DIETARY RATIONS AND REGIMES. Gary H. Wikfors, Jennifer H. Alix, Mark S. Dixon, and Barry C. Smith, NOAA, National Marine Fisheries Service, NEFSC, 212 Rogers Avenue, Milford, CT 06460, USA.

We have conducted feeding experiments with post-set eastern oysters, *Crassostrea virginica*, northern bay scallops, *Argopecten irradians irradians*, and northern quahogs, *Mercenaria mercenaria*, in which we varied quantity and distribution in time of a cultured microalgal feed (mix of two *Tetraselmis* strains) in a factorial grid. Computer-automated molluscan rearing chambers, discontinuously fed downwellers in which shellfish rest on a nylon mesh screen, were used to control feeding parameters. By measuring growth and system feed conversion efficiency (the percentage of microalgal biomass, provided that is incorporated into shellfish soft tissues), we sought feeding rations and regimes that would optimize either growth or conversion efficiency. With this information, feeding strategies based on economic considerations balancing microalgal costs and system-operation costs could be developed for intensive, land based nursery culture of these shellfish.

Results for oysters and scallops were consistent; growth was optimal at a 5% (of shellfish live weight in dry weight algae per day) ration, but conversion efficiency was optimal with a 2% ration, with best results always obtained on the most-frequent feeding regime of $\times 16$ per day. By contrast, quahog clams grew much more slowly on the same rations and regimes of the same microalgal diet, had lower conversion efficiencies, and showed no benefit of more-frequent feeding at rations that supported positive growth. A 10% ration produced the highest growth and conversion efficiencies, but this was the highest ration tested; therefore, the optimum may be higher. From this comparison of oysters, scallops, and clams subjected to identical experimental feeding conditions, we suggest several possible interpretations:

1. Clams require much more food than oysters or scallops
2. The quality of the *Tetraselmis* diet was poor for clams, compared to oysters and scallops
3. Little of the algae was consumed by the clams, because
4. Clam filtration rates are much lower than rates for the other shellfish, or
5. The rearing environment was not appropriate for clams

Support for all of these hypotheses can be found in previous studies. The overall conclusion is that quahogs appear to be poorly suited to intensive, land-based nursery production on the cultured algal diets that work well for oysters and scallops.

PELLETED DRY FEEDS FOR JUVENILE TROPICAL ROCK LOBSTER *PANULIRUS ORNATUS* THAT OUTPERFORM MUSSELS. Kevin C. Williams, David M. Smith, Margaret C. Barclay and Simon J. Irvin, CSIRO Marine Research, 233 Middle Street, Cleveland Queensland 4163, Australia. E-mail: Kevin.williams@csiro.au

On-growing of tropical rock lobster *Panulirus ornatus* from wild seed is a thriving aquaculture industry in SE Asia, notably Vietnam where current annual production is estimated at 2,000 t. Culture relies on feeding small crabs, paddy snail, oysters and fish bycatch. The dwindling supply of these feeds and the downstream impacts of their use are of increasing concern to aquaculturists and environmentalists alike. A cost-effective pelleted feed that enables lobsters to grow rapidly to marketable size is a high priority. Previous studies at this laboratory showed that juvenile *P. ornatus* readily ate dry feed pellets and that growth exhibited a clear dose dependent response to dietary protein content. However, even on the best performing laboratory-made feeds, lobsters grew only at 60% of the rate achieved in the wild. This sub-optimal growth was attributed to a rapid loss of the pelleted feed's attractiveness upon immersion in water. To understand why this attractiveness was lost so quickly, we quantified the nature of chemical loss from immersed feeds and correlated this to the lobster's preference for these feeds.

The leaching loss from thawed frozen green lip mussel *Perna canaliculus* (GM), pelleted feeds (PFs) containing homogenates of either GM, fresh prawn *Metapenaeus bennettiae*, bloodworm *Marpysa sanguinea*, or squid *Sepioteuthis spp* (each included at 50 g kg⁻¹ dry matter (DM)) and a commercial kuruma shrimp feed (KF) were examined after immersion in water for up to 7.5 h. DM loss from GM was 27% for 0–1 h and a further 7% over the next 6.5 h as compared to losses of 7%–10% in the first h and a further 3%–8% in the next 6.5 h for PFs and KF. For the 0–1 h period, loss of Kjeldahl N, trichloroacetic acid-soluble protein (TCA-P) and total free amino acids from GM was from 2.5 to 6-fold greater than from KF with losses from PFs being slightly less. These differences lessened with increased immersion time. PFs and GM were individually preference-tested ($n = 6$) against KF, either after the same pre-soaking period or when non-soaked. Lobsters' preference for PFs and KF was always less than for GM, irrespective of whether or not KF was soaked, and independent of immersion time. Regression analysis identified TCA-P, glycine and taurine as the principal components influencing the lobster's feeding preference.

These results suggested that using a peptide-rich ingredient such as krill hydrolysate might enhance the lobster's consumption of pelleted feeds. This was confirmed by using it at a constant 8% of formulation in two growth assays: an 8-week dietary protein requirement study (five increments and GM; $n = 4$) and a 12-week dietary astaxanthin requirement study (four increments, GM and blue mussel *Mytilus edulis* (BM); $n = 4$). In the first assay

with 1.9 g lobsters, those fed the highest protein feed (61%, DM) grew twice as fast as those fed GM (0.91 and 0.42 g/wk, respectively). In the second assay with 18 g lobsters, those fed the pelleted feeds grew faster than those fed either GM or BM (2.81–3.26, 2.54, and 1.74 g/wk, respectively).

PATTERNS OF REPRODUCTION AND SPAWNING BEHAVIOUR FOR SCALLOPS *PECTEN NOVAEZELANDIAE* IN NORTHEASTERN NEW ZEALAND. James R. Williams and Russell C. Babcock, Leigh Marine Laboratory, University of Auckland, PO Box 349, Warkworth, New Zealand.

Studies of reproduction in free-spawning marine invertebrates usually involve repeated destructive sampling from a population to measure changes in mean gonad condition. This sampling makes it virtually impossible to answer questions such as: Do entire populations spawn synchronously or sequentially in groups? Do individuals spawn repeatedly? Do gonads redevelop between spawnings? Answers to these questions are vital to enable us to accurately estimate total egg production, probability of fertilization, and the timing of spawning in relation to key environmental signals. In the scallop *Pecten novaezelandiae*, the developmental stage of the gonad can also be assessed without harming the animal, making sequential measurements within individuals possible.

In this study, reproduction in scallops was measured in four populations in the Hauraki Gulf, New Zealand from 2000 to 2003. A macroscopic gonad visual grade (VG) was developed and validated by regressing VG with quantitative histological data ($r^2 = 0.80$ – 0.87). Changes in VG, gonad mass, and histology were measured in scallops destructively sampled to describe variation within and among populations. In two of the populations, tagged scallops tethered to the seabed were also monitored and changes in VG were compared to data from destructive samples.

The *in situ* VG data revealed that scallops participated in multiple spawning events within a reproductive season. Spawning synchrony within a patch varied from 0%–75%. Partial spawnings were common, and gonads redeveloped between spawnings. This is the first study to describe the seasonal reproductive behavior of individuals from a non-colonial, free-spawning taxon. Spawning events were correlated with environmental variables to indicate proximal spawning cues, although the timing of spawning varied on a number of spatial and temporal scales. Major spawnings coincided with sharp drops in sea-surface temperature associated with wind-driven upwelling. Temperature change could be a distinctive inducer to synchronize spawning among individuals, and may result in gametes being expelled into water masses favorable for fertilization and larval development.

COOPERATION OF SHELLFISH GROWERS AND BIOLOGISTS IN DETECTING AND MONITORING INVASIVE SPECIES: THE EUROPEAN GREEN CRAB INVASION IN OREGON. Sylvia Behrens Yamada*, Zoology Department, Oregon State University, Corvallis, Oregon 97331-2914. E-mail: yamadas@science.oregonstate.edu

The recent invasion of Pacific Northwest estuaries by the European green crab, *Carcinus maenas*, initially caused much alarm. Following the strong El Niño of 1997–98, young green crabs appeared in estuaries along the coasts of Oregon, Washington, and as far north as Nootka Sound on the west coast of Vancouver Island, British Columbia. Heath Hampel, a shellfish grower, in Coos Bay, Oregon played a critical role in alerting biologists in this invasion, thus allowing us to document and interpret the invasion process. Unusually strong northward-moving coastal currents of up to 50 km/day during strong El Niño events correlated with good recruitment and northward range expansions of green crabs. It thus appears that these currents transported larvae from more established populations in California to Northwest estuaries. Since the last El Niño, coastal transport events have been much weaker and green crab recruitment much lower.

Shellfish growers and sports crab harvesters in Oregon and Washington also help biologists determine the age structure of the green crab population by taking their captured green crabs to extension offices. From carapace width and condition biologists can estimate age. For example, in the summer of 2003, we saw large individuals from the strong 1997–1998 year class decrease in numbers and smaller, younger crabs taking their place.

While green crab abundance in the Northwest is still low, it is imperative to continue monitoring efforts to elucidate the process of range expansion of this non-indigenous marine species and to serve as an early warning system for the next strong recruitment event. However, as the novelty of a marine invader wears off and densities remain low, funding sources for studying the invasion dry up. As this happens, the role of shellfish growers and crab harvesters in reporting non-indigenous species sightings to biologists becomes increasingly important.

THE FARMING OF AND PEARL CULTIVATING FROM WING OYSTER *PTERIA PENGUIN* IN SOUTHERN CHINA. X. Y. Yu, M. F. Wang, F. L. Liang, and Y. Liu, Pearl Research Institute, Zhanjiang Ocean University, Zhanjiang 524025, China; X. Y. Yu, and J. F. Gui, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China. E-mail: yuxyong@163.net

The wing oyster *Pteria penguin* distributed along the coast of Hainan Island, Leizhou Peninsula and other maritime regions in Southern China Sea. It is a kind of large-sized and fast grow-

ing bivalve species. The trial farming of and pearl cultivating from this species were conducted about 5 years ago. At first, natural shells were captured mainly for half-sphered pearl (mabe) culturing, then the matured wild individuals were chosen as parents for seed producing. After a serial of trials, the procedures of the stock shells selecting and cultivating, inducing spawning, larval rearing, spat collecting, and nursing out and fostering them to matured adult was successfully developed. In this disquisition, the whole procedure from zygote to adult was depicted.

Sufficiently proliferation primarily from the wild shells and then from farmed stocks provided enough oysters for trials of pearl producing. Cooperated with our research group, two companies in Hainan Island and one in Leizhou Peninsula have developed technology of producing mabe profitably. Recently, round pearls yielded successfully from this species. Cultivation of half-sphered and round pearl from wing oyster also described generally.

GENETIC ANALYSIS OF SELECTED STRAINS OF THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA* GME-LIN) USING AFLP AND MICROSATELLITE MARKER S. Ziniu Yu and Ximing Guo, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, Port Norris, NJ 08349, USA. E-mail: carlzyu@hsrl.rutgers.edu

Amplified fragment length polymorphisms (AFLPs) and microsatellite markers were used to examine genetic variation and divergence among four selected strains (DBH, NEH, FMF, and CTS) and one wild population (DBW) of the eastern oyster, *Crassostrea virginica* Gmelin. Eighty-six AFLP markers (from three primer sets) and five microsatellite loci were used for the analysis of 30 oysters from each of the five populations. Microsatellites were considerably more variable than AFLPs. Observed heterozygosities ranged from 0.560–0.640 across the populations for microsatellites, and from 0.186–0.207 for AFLPs. Microsatellites detected more genetic variation per locus, whereas AFLPs were more efficient at generating informative loci. Both pairwise F_{st} and Φ_{PT} analysis of microsatellite data and pairwise Φ_{PT} analysis of AFLP data revealed significant genetic differentiation between all pairs of populations. There was no significant reduction in heterozygosity in all four selected strains compared with the wild population. However, the number of alleles per locus (N_a) for microsatellites was lower in the selected strains than that in the wild population, mostly due to the losses of rare alleles. Two strains subjected to long-term selection for disease-resistance showed consistently increased frequencies at a few AFLP loci, which deserve further analysis to determine if they are linked to disease-resistance genes.

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JOURNAL OF SHELLFISH RESEARCH

VOLUME 23, NUMBER 2

AUGUST 2004



The Journal of Shellfish Research
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Volume 23, Number 2

ISSN: 0730-8000

August 2004

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ANTIMICROBIAL ACTIVITY OF COPPER AND ZINC ACCUMULATED IN EASTERN OYSTER AMEBOCYTES

OCT 04 2004

WILLIAM S. FISHER

United States Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, 1 Sabine Island Drive, Gulf Breeze, FL 32561

ABSTRACT The distribution of eastern oysters *Crassostrea virginica* near terrestrial watersheds has led to a general impression that low or variable salinity is imperative for survival. However, freshwater runoff contains numerous mineral elements from geologic deposits that could play significant roles in oyster physiology. Two metals of terrestrial origin, copper and zinc, are accumulated to extremely high concentrations in eastern oysters, even in the absence of anthropogenic sources. As yet, there has been no defensible demonstration of a physiologic function for such high concentrations. Both copper and zinc, however, are accumulated almost exclusively in the amebocytes and calcareous shell of oysters, a unique distribution that implicates a role in the functions of amebocytes. Amebocytes are migratory, diapedetic cells generally recognized to provide nutriment and defense through phagocytosis, killing, and digestion of invading or ingested microorganisms. There is sufficient evidence in existing literature to suggest that copper and zinc directly contribute to these antimicrobial activities. This review presents historical and recent findings that demonstrate a strong affinity of oyster amebocytes for copper and zinc (even in low ambient concentrations), prolonged retention of the metals despite a potential route of elimination, and strong circumstantial evidence of antimicrobial activity by accumulated copper and zinc. It is proposed that oysters actively concentrate copper and zinc as antimicrobial agents to be used in intracellular and extracellular killing (direct toxicity) as well as extracellular clot formation (precipitation of hemolymph). This potential, combined with evidence of amebocyte involvement in deposition of oyster shell, provides an alternative framework for understanding amebocyte functions, defense activities, and coastal distributions of oyster populations. It also affords some resolution to the apparent contradiction of eastern oysters thriving at seemingly polluted locations.

KEY WORDS: oysters, *Crassostrea virginica*, marine bivalves, copper, zinc, metals, contaminants, amebocytes, leucocytes, hemocytes, internal defense, immunomodulation, immunosuppression, metal toxicity, sentinel species

INTRODUCTION

Eastern oysters *Crassostrea virginica* are distributed across the North American coast of the Atlantic Ocean and Gulf of Mexico in bays, estuaries, and coastal zones, most often near sources of fresh water. The proximity to fresh water has often led to a conclusion that oysters require the low or variable salinity provided by freshwater inflow (Cake 1983, Soniat & Brody 1988, Berrigan et al. 1991). Intermittent salinity reductions can deter stenohaline predators such as oyster drills (Loosanoff 1955, Lunz 1955, Hopkins 1956, Wells 1961, Menzel et al. 1966, Berrigan et al. 1991) and can slow incidence of disease caused by the protozoans *Perkinsus marinus* and *Haplosporidium nelsoni* (Sprague et al. 1969, Ford 1985, Ford & Haskin 1988, Chu & Greene 1989, Chu et al. 1993, La Peyre et al. 2003). However, there is ample evidence of near-shore, intertidal oyster beds in high-salinity estuaries and coastal zones (Beaven 1955, Gunter & Geyer 1955, Lunz 1955, Nelson 1955, Copeland & Hoese 1966). It is possible then, that eastern oysters are influenced by other factors that accompany freshwater influx, such as terrestrial elements, rather than reduced salinity. Copper and zinc, like many elements in the marine environment, originate from natural terrestrial sources and are delivered to oyster beds in watershed runoff (Prytherch 1934, O'Connor 2002). Runoff with sufficient volume (i.e., streams and rivers) can generate flows that bring elements far and deep into the receiving water where subtidal oyster beds exist. Without such runoff, oysters may be confined to shallow intertidal areas adjacent to land where the elements originate. If this is correct, the availability of terrestrial elements may well be a principal determinant in the distribution of eastern oysters.

Copper and zinc are terrestrial elements of special interest because they accumulate to extraordinary concentrations in eastern oysters. They are accumulated against chemical gradients, even from low ambient concentrations, and are retained within the oyster longer than other metals, despite the apparent availability of an elimination mechanism. Moreover, zinc and copper are exclusively sequestered in oyster amebocytes (Fig. 1). This brings them into direct contact with a cell type credited with many indispensable responsibilities for oyster survival, including antimicrobial activities for defense and nutrition. These considerations implicate a physiologic reliance on copper and zinc that, if true, would support the concept that terrestrial elements are key factors in oyster success and distribution. Furthermore, it would resolve the apparent contradiction that eastern oysters often thrive at relatively polluted locations (e.g., Abbe & Sanders 1986). Fortunately, there is ample information in the literature to examine such a possibility. Because of the highly accumulated concentrations and unique association with amebocytes, uptake and disposition of copper and zinc in oysters has been widely studied.

Throughout the literature a perception exists that high concentrations of metals, copper in particular, are harmful to oysters. Yet evidence for such a perception is lacking, at least for adult eastern oysters. In fact, the highest known tissue concentrations of copper and zinc for any marine organism are found in tissues of apparently healthy eastern oyster adults. The perception of harm originates from the knowledge that such high concentrations would be quite toxic to most other biota. Yet, eastern oysters not only survive high ambient concentrations of copper and zinc, but avidly recruit the metals and accumulate them without ill effect. Several studies have shown that concentrations in oyster tissues are much higher than in the ambient environment, and that oysters assimilate and retain copper and zinc from environments with low ambient

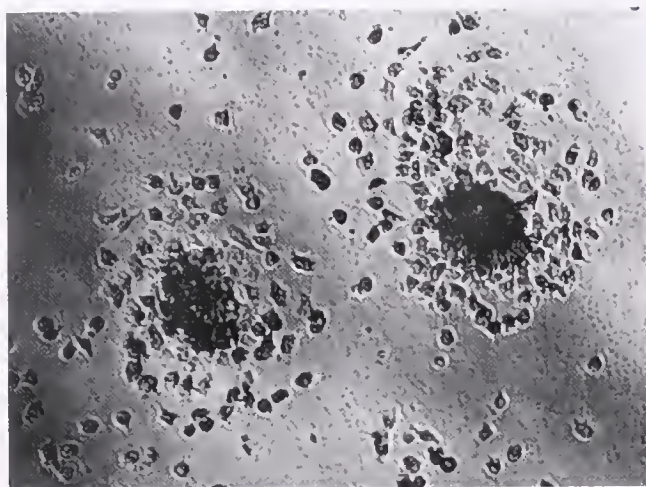


Figure 1. Amebocytes of eastern oysters *Crassostrea virginica* consist of granular and agranular cells capable of movement throughout the hemolymph, tissues, and mantle cavity of the organism. The ability to traverse epithelial layers is called diapadesis. Amebocytes shown here were withdrawn with hemolymph plasma from the adductor muscle and allowed to settle in seawater in a glass dish. Amebocytes form aggregates (clumps of cells) upon withdrawal, but eventually begin to move away from the aggregates using ameboid locomotion.

concentrations. The latter refutes any suspicion that eastern oysters passively, or inadvertently, absorb metals or that they accumulate metals only when exposed to polluted environments. If we accept that successful organisms do not expend energy without purpose, then active uptake and storage implies that oysters must accumulate copper and zinc for a physiologic purpose.

The literature is reviewed here to characterize evidence that supports a physiologic function for copper and zinc as antimicrobial agents in amebocytes. High concentrations are likely required to provide an expansive and flexible defensive and nutritional capability. More specifically, it is hypothesized that they serve as precipitating agents in extracellular clotting and as toxic antimicrobial agents for both extracellular wound protection and intracellular phagocytic activity. It is further speculated that copper, zinc, and possibly other terrestrially derived elements are essential precipitating agents for adult shell deposition (Fisher 2004). Dependence of these activities on copper may have co-evolved with the apparently exclusive requirement for this metal in eastern oyster setting and metamorphosis (Prytherch 1934). Because of these dependencies, availability of copper and other terrestrially derived metals have a major influence on the success and distribution of eastern oysters.

There are several topics relevant to this proposal and an abundance of evidence to consider. A historical perspective (Section I) summarizes early observations of copper and zinc accumulation in oysters, including emergence of a perception that high concentrations of copper are abnormal and toxic to oysters. Section II reviews the close association of copper and zinc with amebocytes, including their exclusive storage in amebocyte granules and positive association with amebocyte numbers. Section III introduces evidence of avid and non-toxic uptake and retention of copper and zinc in granules of eastern oyster amebocytes. Field studies summarized in Section IV demonstrated greater amebocyte numbers and antimicrobial activity for oysters with high tissue concentrations of copper and zinc. In Section V, data are presented that

support a role for copper and zinc in oyster defense activities. Finally, in Section VI, a series of umbrella hypotheses are presented that generate an alternative framework for interpreting high concentrations of copper and zinc, amebocyte activities in nutrition and defense, and the role of terrestrial metals in coastal distributions of eastern oyster populations.

EARLY STUDIES OF COPPER AND ZINC IN OYSTERS

Eastern oysters, once called American oysters, inhabit the coastline along the Atlantic and northern Gulf of Mexico in North America. Scientific names for this estuarine and near-coastal species have included *Ostrea virginiana* (or sometimes *O. virginica*), *Gryphaea virginica*, and the current *Crassostrea virginica*. Although a subsistence food for Native Americans and early colonials, eastern oysters did not represent a substantial commercial interest until the late 19th century (Hargis & Haven 1999). As popular consumption increased, food safety issues grew within the industry. Bacterial and chemical impurities were recognized to cause illness in consumers and, as today, cases of poisoning greatly influenced success in the market.

Green Oysters and Suspected Copper Toxicity

One such chemical impurity was copper. The occasional occurrence of a blue-green tint to oyster meats fueled fears of copper contamination from mines or copper-painted ship bottoms. Oyster tissues resembled the color of copper-salt residues found on cooking vessels (Fig. 2, left). Concerns over green oysters in North America were amplified by the lack of scientific agreement in Europe, where the European flat oyster *Ostrea edulis* had long been a culinary staple. Some asserted that high concentrations of copper were responsible for green *O. edulis* (e.g., Bizio 1845, Thorpe 1896), whereas others believed green color was simply a pigment acquired from ingested food (Gaillon 1824, Thiselton Dyer 1877). The latter group maintained that *O. edulis* raised in holding ponds (*claires*) near the Marennes region of France acquired a green tinge along the gill filaments (see Fig. 2, right) from a diatom *Navicula ostrearia*, which grew in the ponds. The green oysters from Marennes were a highly regarded cuisine.

It is peculiar that a green oyster controversy even existed because O'Shaughnessy (1866) had earlier resolved the conflicting

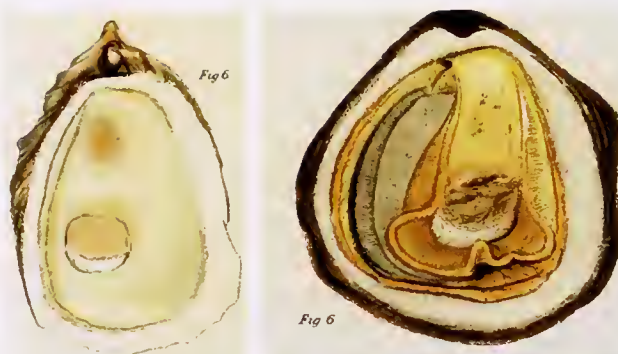


Figure 2. Green-tinted oysters. Left: Eastern oyster *Crassostrea virginica* colored on surfaces of soft tissues by high concentrations of copper in amebocytes. Right: European flat oyster *Ostrea edulis* dyed in the gills by pigment from diatoms (*Navicula ostrearia*). (Drawings reproduced from Herdman & Boyce 1899 in the Lancashire Sea-Fisheries Memoir No. I, Plate VI, Fig 6, and Plate IV, Fig. 6, respectively).

evidence. He related the trial of a tradesman who, "actuated by the lawless desire of premature aggrandizement", was found guilty of selling copper-laden oysters that sickened consumers. The tradesman was unable to obtain green oysters from Marennes, so instead collected substitutes from the outfall of a copper mine in Cornwall, England. O'Shaughnessy (1866) concluded that both diatoms and copper could elicit greenness in oysters. Nonetheless, subsequent investigators in both Europe and America continued to dispute the issue. Some of the dissension is worth recounting because it has fashioned, in part, an existing predisposition that copper is a toxic element for oysters.

Sensitive to the controversy in Europe, John A. Ryder of the Smithsonian Institution reported to the United States Fish Commission that green color in American oysters was most likely a pigment from ingestion of sea cabbage (*Ulva latissima*) or a diatom similar to *Navicula ostrearia* (Ryder 1882). Much of Ryder's conviction however, appears to be based on a notion that oysters could not possibly survive the high concentrations of copper it would take to turn them green.

"That it is not copper we may be equally certain, for any such quantity of a copper salt as would produce the green gills and patches on the mantle, such as are often observed, would without doubt be as fatally poisonous to the oyster as to a human being." (Ryder 1882, p. 410)

Green oysters were tested at two independent laboratories, both substantiating Ryder's claim; there was no evidence of copper in the green portions of tissue. Ryder saw that the green color was actually confined to independent cells that moved freely about the mantle and gills. By virtue of their amebiform ability to migrate, he proposed that these 'blood cells' could enter the digestive tract and absorb green pigment from intestinal juices. But, in spite of his findings, Ryder remained convinced that any oyster with green tissues must be diseased.

Most of Ryder's observations were independently confirmed in studies by Professor E. Ray Lankester (University College, London), who dismissed all reports of copper, at least in those green *O. edulis* that he examined from Europe (Lankester 1886). He strongly believed that green coloration, which he called "marenin", was imparted solely from ingestion of *Navicula*. Like Ryder, Lankester noted that the color was confined to certain "secretion cells" in the gill epithelium. He was puzzled by the ability of these specialized epithelial cells to independently migrate across gill surfaces. Neither Ryder (1882) nor Lankester (1886) were privy to the pioneering insights on invertebrate amoebocytes later summarized by Elie Metchnikoff (1891).

Dr. Thorne Thorpe (1896) determined that "green-bearded" or "green-finned" oysters from Marennes were so-colored from the diatom *Navicula*, and he found that copper quantities in these oysters were sometimes far less than found in "normal" white oysters. Nonetheless, he accepted O'Shaughnessy's contention that greenness could also originate from copper:

"But there is no question that the greenness of certain oysters, especially those found in Falmouth and Truro waters, is due to copper. The color, both in character and distribution, is, however, quite different from that of the Marennes oyster. The green Cornish oyster is unsaleable in this country—at least for immediate consumption—as it leaves a distinct metallic taste in the mouth, similar, it is said, to that due to 'sucking a penny'." (Thorpe 1896, p. 107)

The color, metallic taste and copper content of green oysters gradually dissipated to "normal levels", Thorpe found, with their

transfer to cleaner water. These normal concentrations were judged to be more appropriate for accepted physiologic functions, in particular as a component of the respiratory pigment hemocyanin, which at the time had been described in the blood of octopus and other molluscs (Thorpe 1896).

Professor W. A. Herdman (1896) believed the green tint he observed on experimental *O. edulis* and *O. virginica* was a sign of disease. He ran chemical tests on these oysters and, at first, concluded unequivocally that "copper had nothing to do with the disease" (p. 164). Only 1 year later, however, he reversed his stance to accept the views of O'Shaughnessy and Thorpe.

"There are evidently several kinds of greenness in oysters, and whereas some may be due to normal and healthy processes, others must be regarded as abnormal or diseased conditions. It is the latter, in our experience, that contain the copper." (Boyce & Herdman 1897, p. 31)

Boyce and Herdman (1897) described experiments with *O. virginica* transplanted to Fleetwood, England. They found American oysters had a different shade of green and much higher concentrations of copper than any of the English, Dutch, or Marennes oysters (*O. edulis*), except for occasional green specimens from Falmouth. They recounted several findings: (1) green oysters contained up to four times the copper of white oysters; (2) green tissues contained more copper than white tissues; (3) green coloration was strongly associated with the distribution of green amebocytes (described as ameboid, wandering leukocytes, Fig. 3); and (4) copper was exclusively associated with the green amebocytes. The blue-green tint of the American oysters, they surmised, was due to copper-laden amebocytes that migrated into the mantle cavity and onto the external surfaces of the tissues. They also found tinted amebocyte aggregates in the heart and sinus vessels (Fig. 4), as well as among the connective and epithelial tissues. These findings remain undisputed.

Boyce and Herdman (1897), like Ryder (1882), felt that copper-laden green oysters were diseased. They suggested that copper accumulation in amebocytes was

"...due to a disturbed metabolism, whereby the normal copper of the haemocyanin, which is probably passing through the body in minute amounts, ceases to be removed, and so becomes stored up in certain cells." (Boyce & Herdman 1897, p. 38)

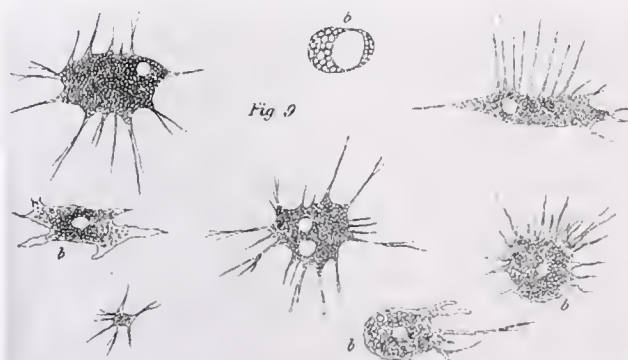


Figure 3. Drawings of large ameboid "leukocytes" found on the surface of gill and mantle of green *Crassostrea virginica*. The presence of granules is evident in the drawings. "b" denotes cells from the blood (hemolymph). (Reproduced from Herdman & Boyce 1899; Plate V, Fig. 9).

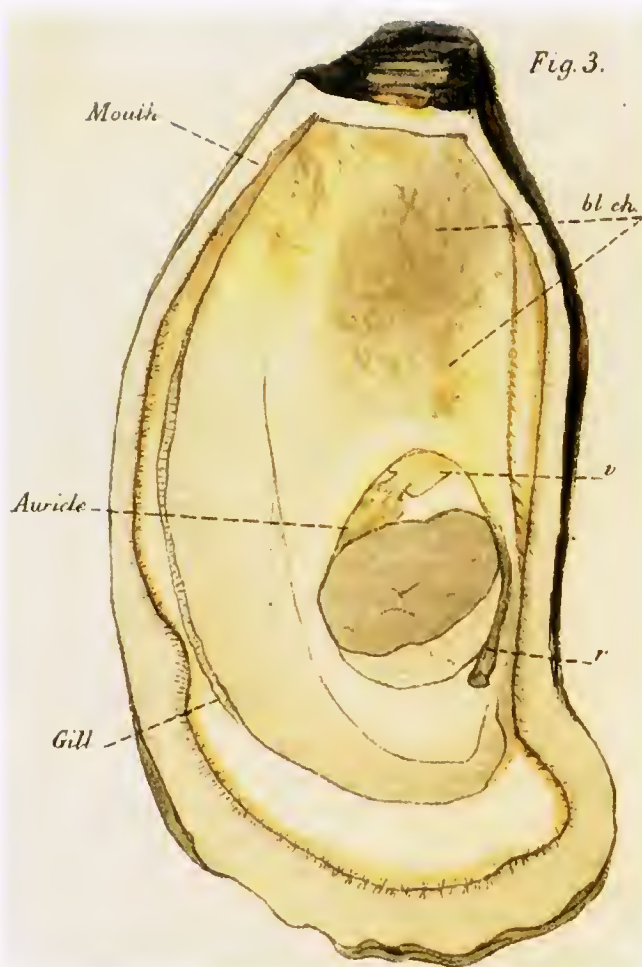


Figure 4. Green-tinted *Crassostrea virginica* showing blood channels (bl. ch.) and auricles of the heart engorged with green amebocytes. The letter v denotes ventricle and r denotes rectum. (Reproduced from Herdman & Boyce 1899; Plate VI, Fig. 3).

They described a degenerative reaction in green oysters, a striking increase in amebocytes that collected in clumps and abnormally distended the blood vessels (see Fig. 4). Their evidence was eventually published in an often-cited treatise (Herdman and Boyce 1899) that clearly distinguished “diseased” American and Falmouth oysters, stained with high concentrations of copper, from the healthy Marennes and Roach River oysters tinted by pigments from diatoms. In their definitive work, Herdman and Boyce (1899) detailed the location and severity of both types of green discoloration with color drawings (see Figs. 2–4), quantified concentrations of copper and iron in oysters from several sources, and reported limited success in staining oysters green through exposure to high concentrations of soluble copper oxide and copper chloride.

In spite of a long history and plentiful evidence, controversy over the role of copper in green oysters persisted into the 20th century (Pease 1911, Nelson 1916 [cited from Hunter & Harrison 1928], Ranson 1924, Ranson 1925, Yonge 1928, Galtsoff & Whipple 1930, Takatsuki 1934). Some confusion may have been due to the fact that a green tint is most easily seen when amebocytes are present on the surface of the tissues, a condition that may vary for reasons unrelated to copper accumulation. For eastern oysters, the controversy was seemingly resolved when Galtsoff and Whipple (1930) found green oysters from New Haven Harbor

(Long Island Sound) to contain copper at concentrations of 1,217–2,719 $\mu\text{g g}^{-1}$ (dry weight) compared with only 82–138 $\mu\text{g g}^{-1}$ in white oysters from Onset Bay (MA). There is now a general acceptance that copper can accumulate in amebocytes to concentrations high enough to impart green coloration. In fact, Galtsoff (1964) noted that,

“In the case of pronounced green discoloration the presence of metallic copper may be demonstrated by inserting in the tissues a well-polished steel knife; the surface becomes copper plated in a short time. This simple method can be used profitably for a qualitative demonstration of the presence of copper.” (Galtsoff 1964, p. 388)

There is no longer any doubt that copper at high concentrations in eastern oysters will create greenish coloration. Abbe and Sanders (1986) predicted that concentrations of copper must be more than 700 $\mu\text{g g}^{-1}$ (dry) for oysters to turn green, and that all oysters would be green at 2,000 $\mu\text{g g}^{-1}$. Along with this acceptance is the unmistakable perception that such high accumulations are abnormal, are a direct consequence of environmental pollution, and are responsible for a toxic or diseased reaction in oysters.

One additional study on the association of copper with eastern oysters is particularly significant. In both field and laboratory studies, Prytherch (1934) found copper was required for setting and metamorphosis of eastern oyster larvae. In studies at Milford, Connecticut, he found larvae would not set if copper was excluded from the seawater. He further showed that by removing copper from the seawater immediately after setting, metamorphosis would cease until its re-addition. Prytherch (1934) cemented oyster larvae to glass dishes to microscopically examine and chronicle morphologic changes during metamorphosis. He observed that larval pigment spots (more often called ‘eye spots’) were composed of about 300 aggregated, immobile amebocytes. Addition of copper to the seawater initiated movement of the pigment spot amebocytes and their inaugural migration into the bloodstream. Although important to this review and pivotal to the argument for amebocyte participation in shell deposition (Fisher 2004), these findings have been generally overlooked in the literature and have never been validated.

High Zinc Accumulation and Radioactive ^{65}Zn from Nuclear Reactors

The earliest reports of zinc accumulation by eastern oysters stemmed from broad-based efforts to determine whether all living organisms contained zinc and, if so, what physiologic purpose the element might serve. Two early contributions (Bradley 1904, Mendel & Bradley 1905) revealed that concentrations of zinc oxide in the hepatopancreas of *Sycotypus canaliculatus*, a large carnivorous gastropod, were well above 10%. They found only negative or “doubtful” concentrations for a variety of other New England marine crustaceans and molluscs, including *O. virginiana*. Mendel and Bradley (1905), based on existing knowledge that many invertebrates contained copper in the respiratory pigment, suggested zinc might bind to an analogous respiratory pigment. Although it was never characterized, they named this presumptive pigment ‘hemocytopyrin’ (Mendel & Bradley 1906, Mendel & Bradley 1907). Zinc was found in the tissues of several marine organisms from the Tortugas by Phillips (1917; Table 1), who concluded it must be a natural constituent because the Tortugas were far removed from anthropogenic influences (although some samples were collected from the moat at Fort Jefferson). This approach, surveying fauna to generate hypotheses of potential function, reached a pinnacle in the work of Vinogradov (1953), who examined metal content in several marine phyla.

TABLE 1.

Concentrations of copper and zinc oxide detected in various marine species from the Dry Tortugas as reported by Phillips (1917). Values were recalculated from analytical results obtained with 20 g dry tissue. Copper was determined electrolytically in nitric acid solution. Zinc was precipitated as a sulphide in acetic acid solution and, weighed as ZnO. Other analytes measured by Phillips (1917) include Fe, MnO, and PbO₂.

Species	Tissue	Copper	Zinc Oxide
		($\mu\text{g g}^{-1}$)	($\mu\text{g g}^{-1}$)
<i>Fasciolaria gigantea</i>	Liver	2,170	180
<i>Fasciolaria gigantea</i>	Liver	3,725	615
<i>Cassis</i> sp.	Liver	350	95
<i>Cassis</i> sp.	Liver	95	70
<i>Strombus bituberculatus</i>	Whole	30	70
<i>Strombus gigas</i>	Liver	30	95
<i>Strombus gigas</i>	Liver	40	380
<i>Strombus gigas</i>	Whole	125	255
<i>Strombus gigas</i>	Whole	10	190
<i>Fulgur perversus</i>	Whole	35	385
<i>Palinurus</i>	Blood	700	None
<i>Palinurus</i>	Liver	900	200
<i>Palinurus</i>	Liver	1,110	190
<i>Limulus polyphemus</i>	Assorted	170	655
<i>Limulus polyphemus</i>	Blood	850	70
<i>Aplysia</i>	Whole	14	55
<i>Aplysia</i>	Whole	15	80
<i>Aplysia</i>	Liver	110	100
<i>Holothuria bermudiana</i>	Muscle	Trace	215
<i>Holothuria bermudiana</i>	Intestines	200	65
<i>Ciona atra</i>	Whole	15	30
Gray tunicate	Whole	200	160

Many early observations on zinc in oysters were focused on the high concentrations that were accumulated. Hiltner and Wichmann (1919) found zinc and copper accumulated to high concentrations in Atlantic coast eastern oysters; they believed the elements were accumulated from the food chain because concentrations were so much greater than for seawater.

"There appears, moreover, to be no direct, uniform ratio between the quantity of copper and zinc in oysters and the amount in the seawater in which they are found, although it is true, in general, that oysters contain larger proportions of the metals when grown in seawater highly contaminated with metallic wastes from smelters and other factories." (Hiltner & Wichmann 1919, p. 217)

Although they noted a potential role for zinc in respiration, Hiltner and Wichmann (1919) attributed no biologic significance to these exceptionally high concentrations. In fact, they concluded that the concentrations seemed too great to even consider a functional role.

Bodansky (1920) examined 20 different marine species from the Gulf of Mexico for accumulation of zinc and found the concentrations in eastern oysters extraordinary. It was, he suggested,

"... a striking phenomenon that the oyster living in a water, not contaminated by industrial wastes, should contain in its tissues more than 35,000 times as much zinc as is present in an equivalent weight of water." (Bodansky 1920, p. 403)

Bodansky (1920) also concluded that the source of zinc was probably oyster food, and he believed that zinc might play a role in respiration. Based on chemical analyses of dissected oyster tissues, he reported that zinc was distributed evenly in the digestive organ, mantle, and gills, yet was considerably lower in the adductor muscle.

Investigations into zinc accumulation by oysters gained significance in the mid-1950s as coastal zones and estuaries were exposed to increasing levels of industrial waste as well as radioactive contamination from nuclear reactors, fuel reprocessing plants, and fallout from intensive nuclear weapons testing. In a study to characterize the radioactive pollution from a nuclear power station in Essex, England, Preston (1968) examined ¹³⁷Cs, ⁶⁰Co, ⁵⁵Fe, and ⁶⁵Zn in the receiving estuary and found that ⁶⁵Zn was the only nuclide appreciably assimilated by oysters. The amount accumulated was proportional to the exposure, as demonstrated by diminishing concentrations of ⁶⁵Zn as oyster collections moved further from the source (Table 2). Because of this capacity to accumulate ⁶⁵Zn and a link with human food consumption, oysters were considered an ideal indicator organism to assess food-borne risk (Murthy et al. 1959, Fitzgerald & Skauen 1963, Wolfe 1970a, Romeril 1971). In general, these reports showed that oysters near radioactive effluents accumulated ⁶⁵Zn at levels too low to affect human health; reports describing effects on oysters have not been found.

Impetus for food safety led to advances in radioactive tracing technology. Methods were developed to track the differential uptake of ⁶⁵Zn by aquatic species and its passage through biogeochemical cycles (Chipman et al. 1958, Watson et al. 1961, Alexander & Rowland 1966, Duke 1967). In one such study, Chipman et al. (1958) showed that clams, scallops, and particularly oysters (*C. virginica*) accumulated zinc thousands of times higher than seawater. Using radioactive ⁶⁵Zn, they found uptake and exchange of zinc between the seawater and tissues to be rapid and in continuous flux. They also noted a considerable amount of ⁶⁵Zn in oyster shells, which they attributed to adsorption because of the dynamic uptake and exchange. Their studies included measurement of uptake by excised oyster gill sections, influences of chelating agents, and tissue distributions of injected ⁶⁵Zn. They attempted to extract ⁶⁵Zn from oyster tissues and found that about half the zinc was extractable by water alone.

Wolfe (1970a) described studies on *C. virginica* from North Carolina that used both stable and isotopic zinc. He found that soft tissues contained about six times the zinc concentration of shells, but because the shells were so heavy they contained nearly 45% of the total zinc in an oyster. Similar to Bodansky (1920), Wolfe found that tissues with large external surface areas (gills, mantle,

TABLE 2.

The amount of ⁶⁵Zn in *O. edulis* tissues decreased with distance from the source, a cooling water outfall from a nuclear generator in Essex, England, as reported by Preston (1966).

Distance from Outfall (Statute Miles)	[⁶⁵ Zn] (pCi/g)
0.33	100
1.0	42
2.0	33
2.75	19
5.75	9.1
6.75	4.5

labial palps, gonad, and digestive gland) had higher concentrations of zinc than other tissues (adductor muscle, pericardial sac). Romeril (1971) demonstrated that *C. angulata* (Portuguese oysters) accumulated ^{65}Zn to higher concentrations than *O. edulis*, but found similar tissue distributions. This fact, he believed, indicated a common uptake mechanism among oyster species. Romeril (1971) found a high concentration of ^{65}Zn with the shell, as had previous investigators (Chipman et al. 1958, Fitzgerald & Skauen 1963, Wolfe 1970a), but showed that co-incubation with iron or cobalt would reduce this ^{65}Zn concentration, presumably due to binding-site competition. This finding supported his conclusion that zinc was adsorbed to the shell and in constant flux.

Few hypotheses on a potential role for such high concentrations of accumulated zinc emerged from these studies. It was clear, since the study of Bodansky (1920), that some zinc was bound to protein and might serve as enzyme co-factors; but calculations by Pequegnat et al. (1969) indicated that accumulation was much greater than any known or speculated requirements. Potential roles for zinc in enzyme activation have been investigated: Zinc-dependent enzymes in *C. virginica* include carbonic anhydrase, alkaline phosphatase and malic dehydrogenase (Wolfe 1970b), and in *O. edulis* they include carbonic anhydrase, alkaline phosphatase, carboxypeptidase A, and α -D-mannosidase (Coombs 1972). However, in both cases the investigators concluded that accumulated zinc was far in excess of enzymatic zinc requirements. This inconsistency between requirement and accumulated concentrations led Wolfe (1970a) to suggest,

"The accumulation of zinc and other trace metals may be partly a coincidental result of poor discrimination by the biologic mechanism for calcium uptake and shell deposition. This possibility is consistent with the observed seasonality of trace element concentration ... where contents of manganese, iron, copper, and zinc were higher during warm months when shell deposition is greatest." (Wolfe 1970a, p. 55)

Considering the great interest that many early investigators placed on a role for copper and zinc in oyster physiology, the lack of ensuing studies is remarkable. This dwindling attention may have risen from a sense that such extraordinarily high concentrations precluded any possible physiologic purpose.

METAL ACCUMULATION IN OYSTER AMEBOCYTES

Ostensibly, eastern oysters flourish in shallow coastal areas and estuaries because freshwater inflow creates reduced or variable salinities that thwart marine predators (e.g., oyster drills; Wells 1961, Menzel et al. 1966) and marine pathogens such as the protozoans *Perkinsus marinus* (Andrews & Ray 1988) and *Haplosporidium nelsoni* (Ford & Haskin 1988). Yet, freshwater inflow also transports terrestrial elements, including both natural and anthropogenic metals, from the watershed. Any dependence by oysters on terrestrial metals must have evolved from natural geologic sources because anthropogenic sources are relatively recent. Nonetheless, the two sources are sometimes confounded. Both metals and polycyclic aromatic hydrocarbons (PAH), even though they are naturally derived, are often associated with human activities because their levels are elevated in urban and industrial discharge (Hiltner & Wichmann 1919, Hunter & Harrison 1928, Chipman et al. 1958, Galtsoff 1964, Pringle et al. 1968, Roosenburg 1969, Boyden & Romeril 1974, O'Connor 2002). Elevated concentrations of metals and PAH, along with wholly anthropogenic polychlorinated biphenyls (PCB) and pesticides, are usually

considered indicators of environmental pollution. Although there are natural sources for metals, a perception persists that certain metals, including copper and zinc, are environmental contaminants with a potential for toxic, detrimental effects on human health and the condition of flora and fauna.

Bivalves as Sentinels of Environmental Pollution

Pesticides, PCBs, PAHs, and metals are the most common chemicals monitored in the coastal systems (O'Connor 2002). These chemicals are generally present in heavily used coastal zones and, accordingly, have been the focus of monitoring programs to determine status and trend of chemical pollution. One of the most comprehensive exposure monitoring programs in the United States, the National Marine Fisheries Service Status and Trends Program Mussel Watch Project, uses soft tissue concentrations in sentinel mussels and oysters to characterize local exposure conditions in relation to other locations (O'Connor & Ehler 1991, O'Connor 2002). Bivalves, presumably because of their filter-feeding behavior, are notorious for their ability to concentrate metals. This characteristic has provided an opportunity to use bivalves to monitor environmental chemicals that are otherwise below detection in the water column or that are highly variable over time.

Bivalve tissue concentrations used in the Mussel Watch Project and other environmental monitoring programs are better used to characterize long-term trends than to estimate actual exposure concentrations (Kopfler & Mayer 1973, Ikuta 1958a, Ikuta 1958b, Roesijadi 1996, O'Connor 2002). Individual bivalves, even when exposed to the same ambient concentrations of chemicals, can accumulate different tissue concentrations. This is shown by the substantial variability among oysters sampled at the same time and location. Some concentrate elements with such effectiveness that they have been termed "super-accumulators" (Lobel & Wright 1983, Wright et al. 1985). Variability in accumulation is often linked to size, age, filtration rate, and reproductive status, but can also vary with environmental factors that include salinity, food availability, and chemical speciation (Chipman et al. 1958, Boyden & Phillips 1981, Phelps & Hetzel 1987, Wright & Zamuda 1987, Bryan & Langston 1992, Roesijadi 1996). Such differences have led to the general conclusion that 15 to 25 oysters (Fig. 5) must be analyzed to provide a representative estimate of metal concentrations for a given site (Kopfler & Mayer 1969, Gordon et al. 1980, Boyden & Phillips 1981, Wright et al. 1985, Sanders et al. 1991, Reidel et al. 1995, Jiann & Presley 1997).

Variable accumulation has confounded interpretations of bivalve tissue analysis and has limited its effectiveness as an indicator of environmental exposure. As Roesijadi (1996) states,

"A consensus on the best approaches for using the metal content of oysters to monitor environmental metal contamination has yet to emerge, although the use of oysters as sentinel organisms is a common activity." (Roesijadi 1996, p. 520)

To offset this variability, monitoring programs often monitor during the same season each year and use animals of similar size. Other approaches include the use of inbred organisms in transplant scenarios (Roesijadi 1996).

High Concentrations of Copper and Zinc are Accumulated in Oysters

Despite uncertainties in interpreting tissue concentrations, evidence supporting zinc as the most prominent element in eastern

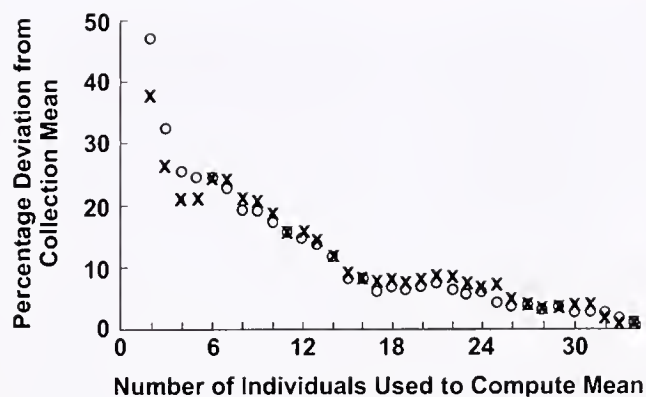


Figure 5. Percentage deviation of the cumulative mean for zinc (X) and copper (O) concentrations from the population mean (defined as 34 animals collected from 9 different sites) determined by Wright et al. (1985) using eastern oysters from Chesapeake Bay. Data from all collections were randomized and then computed as a cumulative mean, starting with two animals and progressing to 34 animals. Data were pooled by ignoring whether deviations were positive or negative.

oyster tissues is overwhelming (Table 3). Zinc is usually followed in quantity by copper (Shuster & Pringle 1969, NOAA 1987) or iron (Lytle & Lytle 1990, Jiann & Presley 1997). Concentrations of zinc invariably comprise 80% or more of the total metal accumulated in eastern oysters, a proportion unique among marine invertebrates (Vinogradov 1953, Pequegnat et al. 1969) and even among filter-feeding bivalves (Pringle et al. 1968, Boyden & Romeril 1974, Boyden 1975). O'Connor (2002) reported that eastern oysters accumulated about 10 times the copper, silver, and zinc of mussels *Mytilus edulis*. This finding is upheld by relatively long-term data collected through the Mussel Watch Project (O'Connor 1996, Table 4), as well as direct comparisons of mussel and oyster collections from the same sites in Long Island Sound (O'Connor 1994) and Chesapeake Bay (Reidel et al. 1995).

Copper has also been found particularly high in oysters relative to other lamellibranchs (Vinogradov 1953, Brooks & Rumsby 1965).

"Except for species of *Ostrea*, all Lamellibranchiata contain copper in quantities not higher than the average amount found in *Gastropoda* . . . species of *Ostrea* have proved to be richest in copper." (Vinogradov 1953, pp. 349–350)

Copper, as described earlier, can be extremely high in oysters exhibiting a green tint. Hiltner and Wichmann (1919) found a mean of 403 μg copper g^{-1} (dry weight) for normal "white" oysters in the northeast United States (see Table 3), but noted that bluish oysters collected from Perth Amboy, NJ, contained 7,435 μg g^{-1} . Similarly, Galtsoff and Whipple (1930) found green oysters to contain 1,954 μg copper g^{-1} , much higher than the mean 109 μg g^{-1} they recorded for white oysters.

Crassostrea virginica may accumulate more copper and zinc than *C. gigas*. Pringle et al. (1968) found from broad geographic surveys that eastern oysters along the Atlantic coast contained 5 to 10 times the zinc and copper as Pacific Coast *C. gigas*. Okazaki and Panietz (1981) found higher accumulations of zinc (but not copper) in *C. virginica* than *C. gigas* at a site in California. Boyden (1975) found *O. edulis* to accumulate more zinc and less copper than *C. gigas* sampled from the same site. Although there is some

TABLE 3.

Examples of copper and zinc concentrations (μg g^{-1} dry weight) reported from tissues of eastern oysters *Crassostrea virginica*.

Mean [Cu]	Mean [Zn]	Source
403 ^a	3,263 ^a	^b cm of 8 sites, Connecticut; Hiltner & Wichman (1919, Table 2)
7,435	14,515	"Blue" oysters, New Jersey; Hiltner & Wichman (1919, Table 3).
219 ^a		^b cm of 5 oysters, Texas; Rose & Bodansky (1920)
	1,299 ^a	^b cm of 5 oysters, Texas; Bodansky (1920)
150 ^a	850 ^a	One eastern oyster; Severy (1923)
109		^b cm of 9 lots of "white" oysters; Galtsoff & Whipple (1930)
1,954		Mean of 6 "green" oysters; Galtsoff & Whipple (1930)
426 ^a		North Atlantic states, winter; Coulson et al. (1932)
85 ^a		South Atlantic states, winter; Coulson et al. (1932)
134 ^a		Gulf States, winter; Coulson et al. (1932)
	6,744 ^a	^b cm from 9 sites; Chipman et al. (1958)
458 ^a	7,140 ^a	Mean of 100 Atlantic Coast sites; Pringle et al. (1968)
95 ^a	1,150 ^a	^b cm from 7 sites in southeast USA; Kopfler & Mayer (1969)
82 ^a	2,969 ^a	^b cm from 8 sites, Mobile, Alabama; Kopfler & Mayer (1973)
60 ^b	6,132	^b cm from 4 sites in Appalachicola Bay, Florida; Magley (1978)
598		Mean of 1978 samples, power plant, deployed; Abbe (1982)
219 ^b	9,188	^b cm of 1978 data, Chesapeake Bay; Wright et al. (1985)
323 ^b	6,701	^b cm of 1979 data, Chesapeake Bay; Wright et al. (1985)
1,480	5,215	Chesapeake Bay power plant, deployed; Abbe & Sanders (1986)
130 ^a	3,020 ^a	^b cm of 5 sites, Mississippi Sound; Lytle & Lytle (1990)
124 ^b	1,950	^b cm from 8 annual geometric means; O'Connor (1996) ^c
310		^b cm of 10 oysters, Chesapeake Bay; Abbe et al. (2000)
415	5,240	Mean of 16 sites, Tampa Bay, Florida; Fisher et al. (2000)
682	5,374	Mean of 22 sites, five Florida Bays; Oliver et al. (2001)

^a Wet weight to dry weight conversion; $\times 5$ (by convention).

^b Calculated mean (cm); average of multiple means from different collections documented in the original study.

^c O'Connor (1996) reported geometric means generated from samples collected annually at 154 sites across the United States from 1986–1993 (8 years).

consistency among reports, inferences from these data must be tempered by recognition of the high variability noted above.

Copper and Zinc Are Accumulated in Granular Amebocytes

Orton (1923) was among the first to measure high concentrations of copper and zinc in oyster amebocytes. During the summer of 1920, *O. edulis* in the commercial oyster beds of the Thames Estuary suffered unusually high mortalities. Because mortality

TABLE 4.

Annual (1986–1993) geometric mean concentrations ($\mu\text{g g}^{-1}$ dry wt) of selected metals in soft tissues of oysters *C. virginica* (composite of 20 for each site) or mussels *Mytilus edulis* (composite of 30 for each site) collected from 154 sites across the US, as reported from the NOAA National Status and Trends Mussel Watch Project (O'Connor 1996). Oysters generally accumulate $>10\times$ the copper and zinc (and silver, O'Connor 2002) accumulated by mussels.

	1986	1987	1988	1989	1990	1991	1992	1993	Grand Mean
Copper									
Mussels	9.9	9.9	9.3	10	8.9	9.0	8.7	8.1	9.2
Oysters	110	110	130	120	150	120	130	120	124 ($\sim 13\times$)
Lead									
Mussels	2.1	2.2	2.1	1.7	1.7	2.1	2.3	1.7	1.9
Oysters	.42	.53	.49	.45	.55	.60	.50	.59	.58 ($\sim 0.3\times$)
Zinc									
Mussels	140	130	130	120	140	130	130	130	131.3
Oysters	1800	1700	2100	2100	2300	1700	2000	1900	1950 ($\sim 15\times$)

events were frequent during 1919 to 1921 in Europe (Italy, France, the Netherlands, Ireland, and in several regions of England), Orton postulated a relationship to wartime contamination, (i.e., hazardous munitions and material strategically dumped or lost from damaged and sunken ships). Accordingly, he examined *O. edulis* for concentrations of trinitrotoluene, nitrites, oil, and metals. Metals in Thames Estuary oysters were low, so metal toxicity was excluded. Yet, his investigations revealed that the amebocytes of oysters could contain high concentrations of copper ($25,900 \mu\text{g g}^{-1}$ dry weight, using $5\times$ wet weight values), zinc ($40,650 \mu\text{g g}^{-1}$) and tin ($2,450 \mu\text{g g}^{-1}$). These concentrations were many times higher than whole animals or sediments from the same location. Orton (1923) inferred from his findings that most, if not all metals in oysters were concentrated in amebocytes.

His investigation led to another intriguing conclusion. When he transplanted metal-laden oysters to environments with low metal concentrations, they eventually eliminated their metal burdens. Rather than steady-state equilibria, he suggested a novel route of elimination.

"It seems possible that metals are excreted by the blood-cells leaving the body of the oyster and carrying the metals with them." (Orton, 1923, p. 17)

Orton's (1923) contributions to the field were thus 3-fold. His studies sustained earlier speculation that green oyster amebocytes had high concentrations of copper; he suggested that zinc, tin, and possibly other metals were retained in amebocytes; and he proposed a novel method for elimination of metals (i.e., exomigration of amebocytes).

The chemical nature of the green pigment in amebocytes was systematically investigated in 1927 when Paul S. Galtsoff and Samuel Lepkowsky applied "microchemical reactions" to paraffin-embedded sections of normal and green-colored American oysters from New England (Galtsoff & Whipple 1930). Treatment with potassium ferrocyanide and hematoxylin confirmed fully the speculations of Herdman and Boyce (1899) that the intensity in green color was in proportion to the copper content in the oyster and that the copper was located almost exclusively in the green leukocytes (amebocytes).

Ultimately, a detailed characterization of both copper and zinc in oyster amebocytes was provided by Craig L. Ruddell (1971) in research published from his doctoral dissertation. He used traditional and novel histochemical staining techniques to locate both

of the metals in membrane-bound granules (lysosomal derivatives) in amebocytes of Pacific oysters, *C. gigas*. Amebocytes laden with copper and zinc were found distributed throughout the hemolymph and tissues. Neither metal was found in any other cell types, including gill and mantle epithelial cells. It seems that some amebocytes (basophilic granular amebocytes, BGA) contained primarily zinc, whereas others (acidophilic granular amebocytes, AGA) contained primarily copper (as presaged by Boyce & Herdman 1897, p. 33). Even so, Ruddell suggested that some amebocytes might store both elements. He did not report on other metals, (e.g., tin [Orton 1923], iron [Galtsoff 1938, Galtsoff 1953] and manganese [Galtsoff 1964]), that might also be retained and transported by amebocytes.

Positive Association of Metal Content with Amebocyte Number and Distribution

Ruddell later hypothesized that concentrations of copper and zinc in different oyster tissues would reflect the tissue distribution of amebocytes. He examined *C. gigas* and *C. virginica* from both contaminated and reference sites and established that a positive correlation existed between the number of tissue amebocytes and tissue concentrations of copper and zinc (Ruddell & Rains 1975). Mantle tissues of oysters from element-rich sites had higher zinc and copper in their amebocytes than those from element-poor sites. In addition, zinc concentrations in the mantle were found linearly and positively associated with the number of BGAs counted in mantle histologic sections (Table 5). This relationship was reinforced by a positive association between BGAs and zinc concentrations derived from examination of mantle (high zinc) and digestive diverticulum (low zinc) tissues from the same oysters. Copper analyses yielded similar trends, but the linear association between amebocytes and copper concentrations was not as distinct as that of zinc. The fact that copper was associated primarily with AGAs rather than BGAs (Ruddell 1971) might have created this difference.

Overall, the results of Ruddell & Rains (1975) established a positive relationship for copper and zinc with amebocyte number and distribution. The metals occurred wherever metal-carrying amebocytes were stationed, sometimes in the adductor muscle and pericardial sac but more often in the gills, mantle, labial palps, gonad, and digestive gland. Ultimately, Ruddell and Rains (1975) used data from both *C. gigas* and *C. virginica* to estimate that

TABLE 5.

Tissue concentrations (dry weight) of zinc and copper were associated with the density (number per unit area) of basophilic amebocytes observed in histological sections of Pacific (*C. gigas*) and eastern (*C. virginica*) oysters by Ruddell Rains (1975), which provides statistical information. Metal analyses were performed on whole oysters, mantle, and digestive diverticulum (dig. div.) tissue.

Sample	Species	Density of Basophils	Tissue Analyzed	[Zn] $\mu\text{g g}^{-1}$	[Cu] $\mu\text{g g}^{-1}$
1	<i>C. gigas</i>	44.5	Whole	336	149
			Mantle	328	129
			Dig. div.	214	61
2	<i>C. gigas</i>	85.4	Whole	422	19
			Mantle	1,039	47
			Dig. div.	400	18
3	<i>C. gigas</i>	189.2	Whole	429	482
			Mantle	1,508	900
4	<i>C. gigas</i>	346.4	Whole	1,656	319
5	<i>C. virginica</i>	213.7	Whole	1,335	290
6	<i>C. virginica</i>	743.0	Whole	4,598	913
			Mantle	5,285	968

nearly all of the zinc (and probably copper) in an oyster was retained within amebocytes.

Localization of Copper and Zinc in Amebocyte Granules

Robert S. Brown (1975), in his doctoral dissertation, further characterized the relationship of copper and zinc with circulating amebocytes of eastern oysters. Using *C. virginica* from Maryland, Brown applied histochemical techniques to show that copper and zinc were localized in membrane-bound intracellular granules. Virtually all intracellular granules stained positive with Mallory's hematoxylin for copper (Fig. 6) or with a fluorescent dye labeled specifically to bind with zinc (Fig. 7). Nongranular portions of cytoplasm and agranular amebocytes were not stained by either chemical. Transmission electron microscopy (unstained sections)

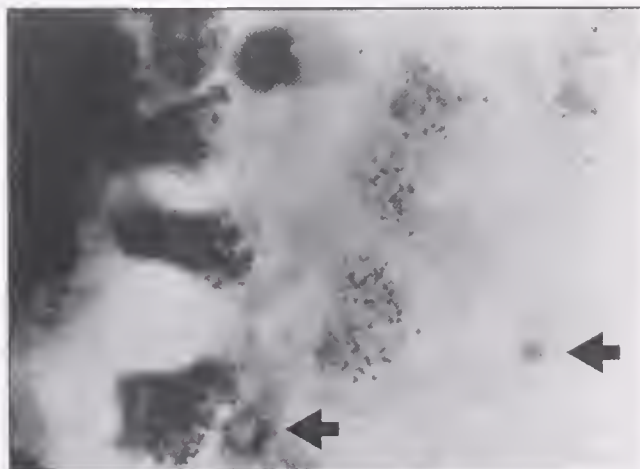


Figure 6. Mallory's hematoxylin stain for copper demonstrates a dark blue color in granular amebocytes of *C. virginica* ($\sim 900\times$). Color occurred in most of the cytoplasmic granules but not in the agranular cytoplasm. Agranular cells (arrows) did not stain positively for copper. (Reproduced from Brown 1975; Fig. 18, with permission of the author).



Figure 7. Zinc demonstrated in *C. virginica* amebocytes using fluorescence of 8-hydroxyquinoline complexes ($\sim 600\times$). An intense greenish-yellow fluorescent color developed within pleomorphic amebocyte granules. (Reproduced from Brown 1975; Fig. 20, with permission of the author).

showed that only the intracellular granules of amebocytes exhibited the electron-dense signature of metals (Fig. 8). High-density material was confined inside the granules, and was not bound by a separate membrane distinct from that of the granule (Fig. 9). Brown concluded that the cytoplasmic granules of amebocytes, the same granules described for *C. gigas* by Ruddell (1971), were storage sites for electron-dense metals.

Brown (1975) was also able to quantify copper and zinc within amebocytes using a relatively novel technique that coupled energy dispersive x-ray analysis to scanning electron microscopy (EDAX-SEM; Fig. 10). Nearly all of the 250 amebocytes probed with EDAX-SEM contained both copper and zinc, although a few cells contained only zinc. The metals were absent from nuclei and from agranular portions of the cytoplasm. Brown determined that *C. virginica* amebocytes could contain as much as 0.3% of their dry weight in copper and 9% of their dry weight in zinc (Table 6). Using EDAX-SEM data, he calculated the content of a single amebocyte to be 6.0×10^{-13} g Cu and 2.5×10^{-11} g Zn, and the content of a single amebocyte granule was 8.5×10^{-15} g Cu and 3.5×10^{-13} g Zn. These calculations assumed 89% granular amebocytes in a sample and 71 granules per cell. Using the same technique, cell-free hemolymph was found to contain copper at an average $0.159 \mu\text{g mL}^{-1}$ and zinc at an average $8.372 \mu\text{g mL}^{-1}$.

These reports of metal accumulation in amebocytes of *C. virginica* led George et al. (1978) to examine "green-sick" *O. edulis* collected from metal-polluted sites in Cornwall, England, as a representative model for studies of bivalve metal detoxification. Their approach included a technique similar to that used by Brown (1975). Using X-ray microprobe analysis (XRP-TEM, a dispersed energy detector coupled to transmission electron microscopy), they located and quantified electron-dense elements within cells. Comparisons of numerous tissues confirmed for *O. edulis* the earlier

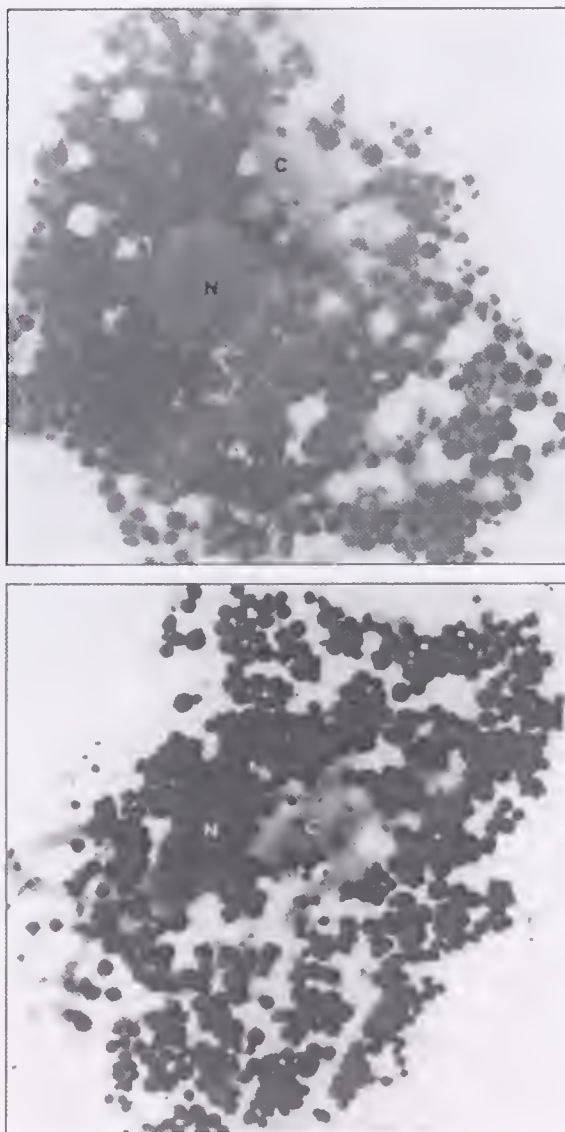


Figure 8. Transmission electron micrographs of unstained granular amebocytes demonstrate the electron opacity (density) of granules in comparison to the nucleus (N) and cytoplasm (C). The top whole-mount was fixed in glutaraldehyde and the bottom was air-dried ($\sim 4000\times$). (Reproduced from Brown 1975; Fig. 21 with permission of the author).

findings on *C. gigas* and *C. virginica*; copper and zinc were localized exclusively within amebocytes. Further confirming the work of Ruddell (1971), George and co-workers found two types of metal-containing amebocytes, BGAs that contained zinc in 1- μm -diameter granules and AGAs that contained copper in 0.8- μm -diameter granules. Analysis of hemolymph and cell-free plasma showed that 70% to 77% of the copper and zinc in the hemolymph was contained in the circulating amebocytes. This estimate, and others comparing hemolymph to cell-free plasma, may well have been influenced by loss of metals from the amebocytes during centrifugation and processing.

Members of the same research team (Pirie et al. 1984) later showed that amebocytes from *O. edulis*, *C. gigas*, and *O. angasi* all had a capacity to accumulate copper and zinc. The latter two species were reported to possess a single cell type that accumu-

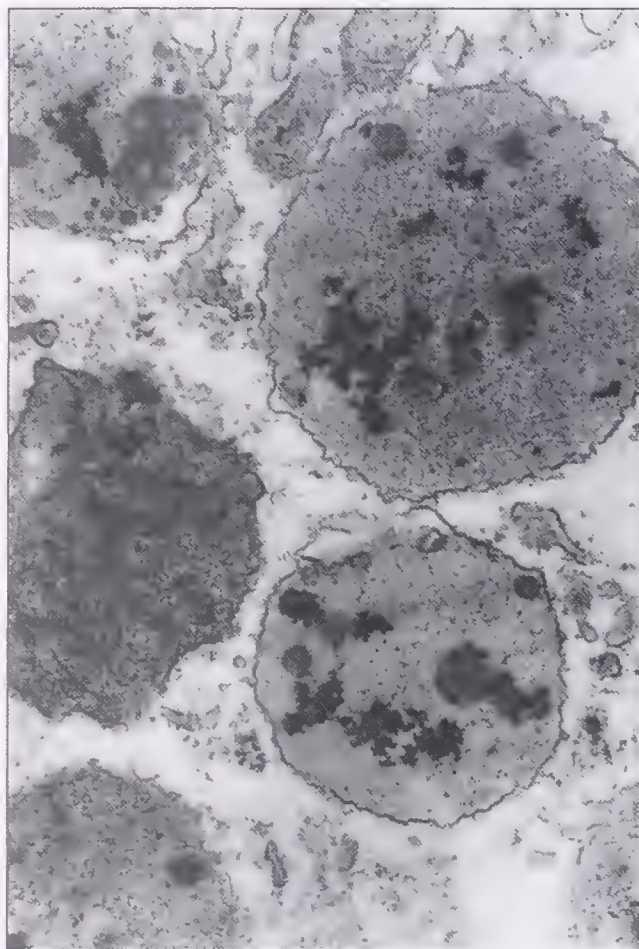


Figure 9. Higher-power transmission electron micrographs provided evidence that the electron dense material of amebocytes resided completely within the membranes of the cytoplasmic granules, shown here ($\sim 86,000\times$). There was no evidence of a separate membrane around the electron dense material. (Reproduced from Brown 1975; Fig. 22, with permission of the author).

lated both copper and zinc, whereas *O. edulis* had copper-specific cells and zinc-specific cells as well as nonspecific copper/zinc (mixed) cells. The metal-specific cells in *O. edulis* were found only from a high-metal environment and mixed cells were from less contaminated sites. The authors acknowledged a possibility that the amount or type of metal present in the environment might have influenced the proportion of different cell types in their studies.

Thomson et al. (1985) compared metals in different cells of *C. gigas* from Tasmania and Wales. Whole body metal analyses showed that oysters from Tasmania contained approximately twice the copper and zinc concentrations of the Welsh specimens, yet XRP-TEM analytical results showed that individual amebocytes from the two groups of animals contained about the same concentrations (see Table 6). This supported the proposition of Ruddell and Rains (1975) that the copper and zinc concentrations were linked to amebocyte number. Thomson et al. (1985) estimated that amebocytes contained $\sim 90\%$ of the copper and zinc in gill and mantle tissues and calculated the concentrations in amebocyte granules to be $506 \mu\text{g copper g}^{-1}$ and $6,375 \mu\text{g zinc g}^{-1}$ (dry weight, converted from mM kg^{-1}), values much less than estimates for *O. edulis* (Orton 1923) and *C. virginica* (Brown 1975).



Figure 10. Analysis of an eastern oyster amebocyte granule using EDAX-SEM microanalysis. Top: scanning electron micrograph of a granular amebocyte ($\sim 3,300\times$) showing the specific granule analyzed (arrow). Bottom: Result of EDAX analysis of an amebocyte granule. X-ray energy (keV) is represented on the x-axis and x-ray emission intensity on the y-axis. Emission detection was greatest at 8.0 and 8.6 keV, corresponding to copper and zinc, respectively. (Reproduced from Brown 1975; Figs. 25 and 26, with permission of the author).

ACTIVE, CONTROLLED UPTAKE AND RETENTION OF COPPER AND ZINC

Copper and zinc accumulation in eastern oysters is so great that many have dismissed the possibility of a physiologic role (Hiltner & Wichmann 1919, Pequegnat et al. 1969, Wolfe 1970a). For copper, at least, high accumulation has even been related to toxicity or disease (Ryder 1882, Herdman 1896, Boyce & Herdman 1897, Korringa 1952). This has led to an overriding impression that copper and zinc are in excess and sequestered in oyster amebocytes solely to prevent toxicity.

"Iron, copper, and zinc maybe be stored in the tissues and in some blood cells as excess materials which are slowly eliminated." (Galtsoff 1964, p. 390)

Simkiss & Mason (1983) introduced three theories to explain the magnitude of copper and zinc deposits in oyster amebocytes: (1) Metals are phagocytosed as foreign material by amebocytes but, being resistant to digestion, are accumulated in the cytoplasm; (2) amebocytes are a specific detoxification system that remove metal ions from the hemolymph to keep concentrations below toxic levels; (3) amebocytes accept metals from other cells (e.g.,

gill cells) to transport them through the blood stream to other tissues (e.g., kidney) for storage and eventual excretion. Yet, to the authors, none of these theories were totally satisfying:

"Many of the more easily detected accumulations of metals in molluscs are tissue specific and they are often associated with particular deposits of granules. These are almost universally interpreted as detoxification or excretory systems, but there is as yet virtually no acceptable experimental evidence for such a conclusion." (Simkiss & Mason 1983, pp. 154-155)

In fact, for eastern oysters, any perception that copper and zinc are sequestered simply for eventual elimination is confounded by an assortment of existing data. Eastern oysters do not seem harmed by high concentrations of copper or zinc in the ambient environment or by high concentrations accumulated in their tissues. Rather, they seem to accumulate copper and zinc only when they have the amebocyte capacity to safely do so. When amebocytes are available, oysters can accumulate copper and zinc from low ambient concentrations and retain them even though effective elimination mechanisms are available. These considerations, described below, not only refute a perception that copper and zinc are sequestered solely for detoxification and elimination, but sustain a contingency that they are stored for a physiologic purpose.

Oysters Concentrate Copper and Zinc from Low Ambient Water Concentrations

Comparisons of oyster soft tissue concentrations with ambient water concentrations of copper and zinc have clearly demonstrated preferential accumulation (Korringa 1952, Boyden & Romeril 1974, Boyden 1975, Simkiss et al. 1982, Roesijadi 1996). Hunter & Harrison (1928) found high concentrations of copper and zinc in eastern oysters had no direct relationship to body weight, other metals, or concentrations in the seawater. They concluded,

"There is reason to believe that oysters will absorb from the water almost any substance which it contains." (Hunter & Harrison 1928, p. 9)

Moreover, it was apparent from even the earliest reports (e.g., Boyce & Herdman 1897) that these metals could be accumulated from metal-poor environments. Measured tissue concentrations have been so high that active, selective uptake seemed inevitable.

"The high proportions of zinc and copper in oysters from beds in the vicinity of industrial plants using these metals can be readily accounted for. The high zinc content of those from beds far removed from any known source of metallic contamination may be explained by the probability that oysters gradually remove traces of the metals from the water and store them in their tissues." (Hunter & Harrison 1928, p. 8).

There is evidence that zinc is more avidly concentrated at low ambient water concentrations than at high. Chipman et al. (1958) compared zinc concentrations of eastern oysters and seawater from several locations along the US Atlantic seaboard. Zinc content was higher in oysters from locations with high ambient zinc, but concentration factors were five times higher at the lowest ambient concentration (Table 7). Preston (1966) estimated a 10^5 concentration factor for zinc. Copper is also concentrated from ambient waters (Ikuta 1958a, 1958b, Pringle et al. 1968, Shuster & Pringle 1969, Kopfler & Mayer 1973). Roesijadi (1996) concluded that uptake of anthropogenic copper and zinc is superimposed on an

TABLE 6.

Concentrations ($\mu\text{g g}^{-1}$ dry weight) of copper and zinc in amebocytes reported for various oyster species. Ruddell and Rains (1975) estimated zinc comprised 6% of the amebocyte cell weight in *C. virginica*. Brown (1975) estimated zinc to be 9% and copper 0.3% of the cell weight. For *O. edulis*, Orton (1923) estimated that zinc comprised 4.1% and copper 2.6% of the amebocyte cell weight. Thompson et al. (1985) estimated that mantle amebocytes retained 93% of the copper and 85% of the zinc in the mantle, and that gill amebocytes retained 90% of the copper and 90% of the zinc in gill tissues.

Species	Source	Method	Cell Type	Amebocyte [Cu]	Amebocyte [Zn]
<i>C. virginica</i>	Ruddell Rains 1975	Histo, AAS	BGA		57,447–63,227
	Brown 1975	AAS		448–2,784	20,548–89,676
<i>C. gigas</i>	Pirie et al. 1984	XRP-TEM	'Mixed'	211 ^{a,b}	1,156 ^{a,b}
	Thompson et al. 1985	XRP-TEM		211 ^{a,b}	1,228 ^{a,b}
<i>O. edulis</i>	Orton 1923	?		25,900 ^b	40,650 ^b
	George et al. 1978	AAS		^b 5,571–65,000 ^b	49,500–125,000 ^b
	Pirie et al. 1984	XRP-TEM	'Cu' cell	900 ^{a,b}	28 ^{a,b}
			'Zn' cell	69 ^{a,b}	3,079 ^{a,b}
<i>O. angasi</i>	Pirie et al. 1984	XRP-TEM	'Mixed'	991 ^{a,b}	703 ^{a,b}
				660 ^{a,b}	2,469 ^{a,b}

^a X-ray probe data; mM/kg converted to $\mu\text{g g}^{-1}$ (multiply by 0.6 for Cu and 0.625 for Zn).

^b Wet weight to dry weight conversion; $\times 5$ (by convention).

Histo, histochemical; BGA, basophilic granular amebocyte; AAS, atomic absorption spectrophotometry; XRP, X-ray probe microanalysis.

already strong natural proclivity. This capacity demonstrates the frailty of tissue analyses to detect pollution, and refutes any perception that metal uptake is passive and indiscriminate.

It is re-emphasized that the substantial concentrations of zinc and copper in eastern oysters are almost exclusively confined to the granules of amebocytes (Section II). Zinc and copper distribution in oysters is thus determined by the number and distribution of amebocytes in different tissues (Ruddell & Rains 1975) and their individual capacity to retain the elements in granules. Concentrations of copper and zinc are high in oysters, higher in granular amebocytes, and even higher in amebocyte granules, underscoring the conclusion of George et al. (1978) that copper and zinc are actively recruited into granules against a strong chemical gradient. Even so, there has been no published investigation of the mechanism of uptake and no evidence of metabolic cost. Galtsoff and Whipple (1930) compared oxygen consumption rates of 'normal' eastern oysters from Onset Bay (MA) with green eastern oysters from New Haven Harbor (CT). Results showed a slight increase in oxygen consumption by green oysters, but the significance was

considered doubtful, based partly on the high variability attributed to muscular activity, gill ciliary activity, and season. Regardless, there seems little doubt that oysters must expend energy to assimilate and retain these metals, especially when they are scarce in the ambient environment.

Logarithmic Uptake of Copper and Zinc in Eastern Oysters

Metal content in tissues of bivalves and other organisms at any given time is the difference between two dynamic processes, influx (uptake rate) and efflux (release rate) (Roesijadi 1996). Changes in influx and efflux rates can be estimated by transferring organisms from low to high (to estimate influx) or high to low (to estimate efflux) exposure concentrations. The number of studies that examine influx outweigh those that examine efflux, but both are equally important in the ultimate disposition of metals in oysters.

Giann and Presley (1997) suggested that, in general, metals accumulate in oysters because of rapid uptake and slow elimination. Whereas rapid uptake may be accurate for most metals, copper and zinc exhibit a unique pattern. Shuster and Pringle (1969) continuously exposed eastern oysters in the laboratory to two elevated concentrations each of Cd, Cr, Cu, or Zn and followed their accumulation in soft tissues over a 20-week period. Upon exposure, cadmium and chromium showed an immediate spike in tissue concentrations that eventually slowed, presumably because they approached a steady state between influx and efflux (Fig. 11). The rate of uptake for copper and zinc, however, did not spike appreciably upon exposure. Instead, a consistent, logarithmic increase in tissue residues was maintained, without decline, throughout the study. Shuster and Pringle (1969) assigned these unexpected results to high initial tissue concentrations. More likely, the two accumulation patterns distinguished two different mechanisms of uptake. Cadmium and chromium exhibited a phased pattern typical of direct tissue absorption whereas the pattern for copper and zinc imply a controlled assimilation that might explain why oysters held in high ambient concentrations do not necessarily have high body burdens (Reidel et al. 1995).

Controlled uptake may be a consequence of the need to seques-

TABLE 7.

Zinc concentration factors of *C. virginica* at various sites in the US calculated from data of Chipman et al. (1958). Lower concentrations of zinc in the seawater coincided with lower zinc concentrations in the tissues, but higher concentration factors. Although not calculated by Chipman et al. (1958), this association was observed by both Preston (1966) and Wolfe (1970a). (Note: oyster tissue data is normalized to wet weight).

Location	Seawater (ng g^{-1})	Oysters ($\mu\text{g g}^{-1}$ wet)	Concentration Factor
Chesapeake Bay	24.0	2933	$\times 122,208$
Milford, CT	18.8	3174	$\times 168,830$
James River, VA	7.9	1484	$\times 187,848$
Beaufort, NC	4.6	1171	$\times 254,565$
Brunswick, GA	1.1	313	$\times 284,545$
Pensacola, FL	0.8	600	$\times 750,000$

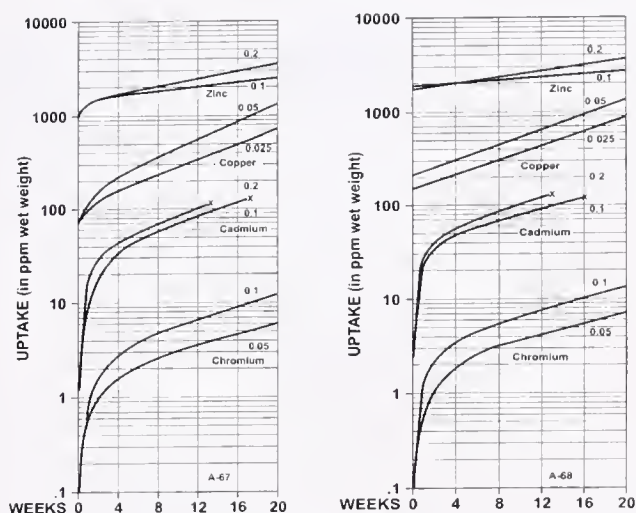


Figure 11. Results of two experiments showing the accumulation of chromium, cadmium, copper, and zinc in eastern oysters maintained in a continuous exposure system for 20 wk. Two concentrations of each element were constantly added to natural seawater from Narragansett Bay, Rhode Island. Experiment A-67, represented on the left, was described in Pringle et al. (1968). Experiment A-68, on the right, was described in Shuster and Pringle (1969). Cadmium exposures were curtailed before wk 20 (X) due to high mortalities. (Figures redrawn from Shuster and Pringle 1969; Figs. 2 and 3).

ter copper and zinc in amebocyte granules. Only a limited number of granules exist in an amebocyte (~71 granules, as estimated by Feng et al. 1971), so there is a limited amount of each element that can be retained (amebocyte metal-carrying capacity). Additional uptake of copper and zinc from ambient sources would be limited until new cells become available. The logarithmic rate of accumulation observed by Shuster & Pringle (1969) seems consistent with this supposition; although cell proliferation has not been well characterized, new cells are probably produced at a logarithmic rate. The fact that uptake might be controlled by a physiologic condition (i.e., the availability of amebocytes with metal-carrying capacity), challenges any perception that copper and zinc are indiscriminately absorbed from metal-rich environments. Rather, it seems that they are actively incorporated when (and only when) amebocytes with metal-carrying capacity are available. Once this capacity is exceeded, further accumulation requires recruitment of new amebocytes, either through proliferation (i.e., hemopoiesis) or activation of existing amebocytes.

Slow Elimination of Copper and Zinc by Eastern Oysters

Just as transfer of oysters from a reference to contaminated site can be used to estimate influx, the converse can provide insight to efflux, or elimination of chemicals. By transferring eastern and Pacific oysters in California from a contaminated to a reference site, Okazaki and Panietz (1981) estimated efflux of various metals. They monitored the loss of metals over a 56-d period from the mantle, gill, digestive gland and kidney, and calculated biologic half-lives ($B_{1/2}$) for each tissue. Half-lives for all four metals tested (Ag, Cu, Hg, Zn) were particularly long for eastern oysters (Table 8), and generally 5× longer than those for *C. gigas*. Ruddell and Rains (1975) had earlier found higher concentrations of copper and zinc and higher numbers of basophils in *C. virginica* than in *C. gigas* from these same two sites. It seems that eastern oysters retain

TABLE 8.

Biological half lives of selected metals as calculated by Okazaki and Panietz (1981) from concentrations in specific tissues of *C. virginica* and *C. gigas* removed from a contaminated site (Redwood Creek) and deployed for 56 d at a reference site (Tomales Bay) in California.

	Tissue Concentrations ($\mu\text{g g}^{-1}$ dry)		Biological Half-Life (d)
	Contaminated	Reference	
<i>C. virginica</i>			
Ag	143	9.5	149.1
Cu	1,548	438	156.2
Hg	3.36	0.38	133.5
Zn	14,365	5,490	183.9
<i>C. gigas</i>			
Ag	162	3.2	26.4
Cu	1,504	93	32.9
Hg	4.26	0.24	23.3
Zn	5,531	474	36.7

^a Average of concentrations determined from mantle, gill, digestive gland and kidney on day 0 from both sites (25 oysters).

^b Average determined from estimates of half-life in separate tissues (mantle, gill, digestive gland and kidney) of oysters transplanted from the contaminated to the reference site for 56 d (5 groups of 5 oysters).

metals for a relatively long period, and significantly longer than Pacific oysters.

Other studies have reached similar conclusions. Zaroogian (1979) collected oysters from Long Island Sound (New York) and held them in seawater troughs for 56 weeks to examine depuration of copper and cadmium. Copper concentrations in the troughs ranged from 1–2 $\mu\text{g L}^{-1}$. He found no statistically significant decrease in copper during this time, and actually noted an increasing trend. Greig and Wenzloff (1978) transferred eastern oysters from a contaminated site (Housatonic River, CT, USA) to an uncontaminated site (Beaufort, NC, USA) and found silver, cadmium, and zinc did not substantially decrease during 40 weeks, and copper was marginally decreased on only one (27-wk) sampling date.

Slow efflux of copper and zinc from eastern oysters indicates that, because even though amebocytes are proficient at detoxifying copper and zinc (i.e., sequestering large amounts in membrane-bound granules), they are strikingly inefficient at eliminating them. This inability is not for lack of a physiologic mechanism. The most likely means for oysters to eliminate copper and zinc is amebocyte exomigration.

"Amebocytes seem to have an important phagocytic role during excretion in molluscs, particularly in eliminating insoluble particles from the circulation. The wandering amebocytes phagocytose the particles and migrate to the gut, the pericardium, the excretory organs or the mantle cavity; they either move out of the body through these viscera or return after releasing the particles." (Narain 1973, p. 8)

This is, of course, the same elimination mechanism anticipated by Orton (1923). Yonge (1928) and others who observed exomigration of green, copper-bearing amebocytes in oyster pseudofeces. Since then, amebocyte exomigration has been described as a means for oysters to eliminate a variety of ingested materials, including india ink, carmine dye, neutral red, aniline oils, coal tar,

iron, and fluorescent beads (Takatsuki 1934, Ranson 1936, Stauber 1950, Galtsoff 1953, Ruddell 1971). In one report, Galtsoff (1953) suggested that iron was actively ingested and then eliminated by amebocytes passing across the mucous cells of the gill and mantle. Potts (1967) considered the evidence for amebocytic elimination strong enough to permit an analogy of these cells with the reticulo-endothelial system of vertebrates.

A description of amebocyte exomigration in *C. virginica* was provided by Stauber (1950), who injected india ink into oyster hearts and collected samples over a 42-day period for gross and microscopic examination.

"The ink suspensions agglomerated readily and produced emboli which virtually occluded the arterial vessels of viscera, mantle and adductor muscle. Subsequent events, with considerable overlapping, were in sequence: (a) phagocytosis of the injected ink particles by mobile phagocytes, (b) distribution of the ink in the phagocytic amebocytes to all parts of the organism with concomitant resolution of the emboli and (c) eventual elimination of the ink from the organism by the migration of ink-laden phagocytes through the epithelial layers of the alimentary tract, digestive diverticula, palps, mantle, heart and pericardium into lumina from which they were voided." (Stauber 1950, pp. 239–240)

The presence of ink-darkened 'dejecta and rejecta' supported Stauber's conclusion that the bulk of the injected ink was eliminated by migration of amebocytes into lumina that opened to the outside of the oyster. A similar process had been described for *O. edulis* by Takatsuki (1934), who showed that carmine particles injected into the body of the oyster were ingested by amebocytes and then distributed into excretory tubules, pericardial epithelium, gonoducts, rectum, mantle cavity and blood vessels. The presence of carmine-containing amebocytes in the mantle cavity provided evidence that they traversed epithelial layers to be discharged in the pseudofeces.

A mechanism is thus available to eastern oysters to eliminate metals and other substances but, by all indications, is not used for copper and zinc. Both metals are retained in the oyster amebocytes at high concentrations for periods well beyond that needed for exomigration. In all likelihood, retention of copper and zinc exceeds many times over the life span of an individual amebocyte, implying that the elements are re-incorporated into new cells. This is probably achieved through phagocytosis of damaged and dead amebocytes (Scro & Ford 1990), and may include apoptosis, wherein damaged and senescent amebocytes pinch off cytoplasmic fragments that are ingested by younger amebocytes without an inflammatory response (Sanderson 1982, Sunila & LaBanca 2003). Metals sequestered in amebocyte granules could be retained through numerous amebocyte cell cycles.

No Evidence of Lethal Copper or Zinc Toxicity to Eastern Oyster Adults

The sensitivity of embryonic and larval stages of *C. virginica* to water-borne metal toxicity has been well documented. Results of 2-day embryo tests (Calabrese et al. 1973) showed copper to be 100% lethal to eastern oyster embryos at $130 \mu\text{g L}^{-1}$ water concentrations ($\text{LC}_{50} = 103 \mu\text{g L}^{-1}$) and zinc to be 100% lethal at $500 \mu\text{g L}^{-1}$ ($\text{LC}_{50} = 310 \mu\text{g L}^{-1}$). In a subsequent study, MacInnes (1980–81) demonstrated a synergistic toxicity for copper and zinc in eastern oyster embryos at concentrations of 8–16 $\mu\text{g Cu L}^{-1}$ and 100–200 $\mu\text{g Zn L}^{-1}$. For larvae of *C. virginica*, Calabrese et al. (1977) estimated lethal copper toxicity for a 12-day exposure

as $\text{LC}_{50} = 32.8 \mu\text{g L}^{-1}$, with growth of surviving larvae reduced by 30%. Prytherch (1934) noted that larval exposure to very high copper ($>800 \mu\text{g L}^{-1}$) would prove lethal even with very short exposure periods (minutes to hours).

In contrast, there is little evidence of water-borne toxicity to adult oysters. One early study attempted to characterize anticipated toxic effects:

"In the first place, we tried the effect of pieces of copper, copper filings, and copper dust lying in the bottom of the aquarium; and similarly, of steel filings, old rusty nails, and other fragments of iron. We also kept oysters for some time in an old copper vessel, and along with copper pyrites and other ores of copper. None of these gave any definite result." (Herdman & Boyce 1899, p. 32)

In subsequent experiments however, they exposed eastern oysters to 50 grains of copper oxide in a gallon of water, which nominally resulted in some green coloration and some mortality within a few weeks. Other than this unsubstantial report, there have been no definitive demonstrations of lethality for copper or zinc exposures to adult eastern oysters. This is underscored by O'Connor (2002), who reviewed a large data set compiled by Jarinen and Ankley (1999) on chemical effects to survival, growth, and reproduction of aquatic organisms; there were no data relating lethal effects of copper or zinc to adult eastern oysters. This intriguing lack of evidence led Roesijadi (1996) to conclude that adult eastern oysters are tolerant of high copper and zinc concentrations in the water column and in their tissues.

There is some published evidence of "negative" toxicity test results. In two carefully controlled, continuous-exposure experiments (Shuster & Pringle 1969), there were no lethal consequences to eastern oysters exposed to copper and zinc, even though tissue concentrations were significantly elevated over the 20-week exposure (see Fig. 11). Cadmium (100 and 200 $\mu\text{g L}^{-1}$) caused significant mortalities, but not copper (25 and 50 $\mu\text{g L}^{-1}$), zinc (100 and 200 $\mu\text{g L}^{-1}$), or chromium (50 and 100 $\mu\text{g L}^{-1}$). Copper accumulated in the oyster tissues to over 5000 $\mu\text{g g}^{-1}$ (dry wt) and zinc to over 15,000 $\mu\text{g g}^{-1}$. There are, furthermore, numerous examples of extremely high concentrations of copper and zinc accumulated in apparently healthy eastern oysters (see Table 3). Frazier (1975, 1976) found adult *C. virginica* survived elevated concentrations of copper in experimental studies, but the shells deposited during this time were thinner and weaker. Okazaki (1976) showed copper toxicity to adult *C. gigas*, but at very high concentrations (96-h median tolerance limit = 560 $\mu\text{g L}^{-1}$) so this species may also be relatively tolerant.

It is not clear how sequestration in granules prevents copper and zinc toxicity. The membranous lining of the granules may shield vulnerable tissues, or the metals may be complexed within the granules so they are unavailable to vulnerable tissues. As long as additional uptake requires generation of new amebocytes and their protective granules, elevated ambient water concentrations will not lead to oyster toxicity. However, this mechanism relies on the ability of amebocytes to capture incoming (ambient) copper and zinc before contact with vulnerable tissues. Most likely, this phase is achieved through binding of the metals to mucus in the mantle cavity, as proposed below.

Mucus Capture of Ambient Copper and Zinc

Any water-borne or food-borne element entering an oyster first encounters the copious mucus covering all external surfaces of the soft tissues (Fig. 12). Hillman (1968, 1969) investigated cellular

ALTERNATIVE FATES OF CHEMICALS IN BIVALVES

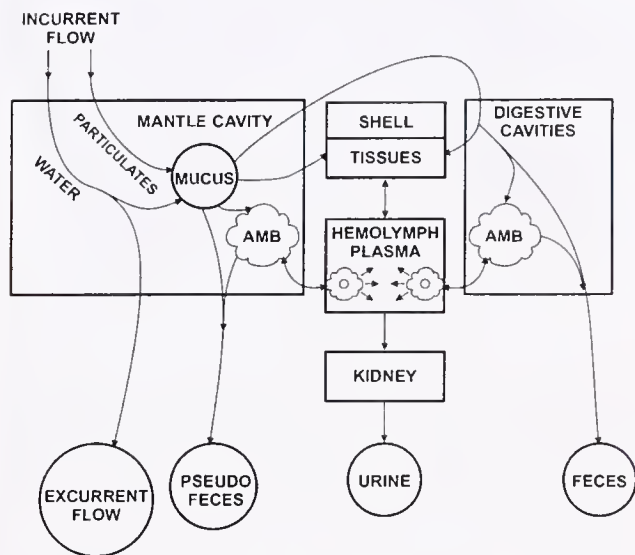


Figure 12. Schematic diagram of potential fates for incurrent soluble and insoluble materials in oysters. Amebocytes (AMB) are capable of moving throughout the oyster with material or abandoning the oyster (exomigration) to carry away unwanted particles in the pseudofeces or feces. Amebocytes in the digestive cavities are believed to play a primary role in nutrition through phagocytosis of incoming particles. (This diagram was based on a model for zinc dynamics published by George and Pirie 1980; Fig. 8).

and compositional differences of mucus in *Mercenaria mercenaria* and concluded that mucus was, "playing far more sophisticated roles in the life activity of the clam than previously suspected" (Hillman 1978, p. 21). These roles include organismal protection, lubrication, and food capture. Mucus of bivalves contains complex carbohydrate sulphates that operate as an ion-exchange mechanism to capture and retain particles and elements (Pringle et al. 1968), especially divalent cations such as copper and zinc (Korringa 1952). If toxic metals were bound to mucus, then they would no longer be available for direct absorption into vulnerable tissues. Instead, they would flow with the mucus sheets across tissue surfaces toward the mouth for elimination (pseudofeces) or ingestion (alimentary tract). This is a common path for selection of oyster food particles (Loosanoff 1949, Menzel 1955); eastern oysters are capable of preferentially ingesting organic particles while rejecting inorganic particles with the pseudofeces (Newell & Jordan 1983). If bound to mucus, water-borne copper and zinc entering the mantle cavity could be eliminated without ever contacting vulnerable tissues.

Accumulation, however, could still be achieved through amebocyte phagocytosis of metal-laden mucus and metal-laden food particles trapped in the mucus. Through diapedesis, amebocytes enter both the mantle cavity and the alimentary tract to capture mucus and food. The process of phagocytosis includes formation of phagosomes, cytoplasmic inclusions (Section V) that exhibit membrane structures similar to copper- and zinc-bearing granules. Metals might be retained in the phagosome after food particles and mucus are digested. If so, phagosomes may be the precursors to cytoplasmic granules. Other possibilities for uptake of metals exist. In particular, vertebrate phagocytes have displayed natural re-

sistance-associated macrophage protein (nramp) activity that transports metal ions for defensive purposes (Atkinson & Barton 1998, Jabado et al. 2000). It is believed that nramp proteins remove divalent cations from phagosomes, thereby depleting phagocytosed microorganisms of essential elements. Accumulation or retention of metals in oyster amebocyte granules might use a similar, albeit inverted, process.

A Presumptive Biologic Function for Copper and Zinc

Information has been presented to dispel any perception that copper and zinc are sequestered by oysters solely to avoid toxicity. Amebocytes acquire high concentrations of copper and zinc from low ambient concentrations and retain them for relatively long periods despite the potential for elimination by exomigration. Retention of copper and zinc may exceed the life span of an individual amebocyte. The lack of toxicity to eastern oyster adults, even at high water and high tissue concentrations, is most likely attributable to binding of the metals by mucus and sequestration in membrane-lined granules of amebocytes. These findings support speculation that eastern oysters store high amounts of copper and zinc for eventual use in critical physiologic functions. The following section provides evidence that antimicrobial activity is one of those functions.

HEIGHTENED AMEBOCYTE ACTIVITIES AT CONTAMINATED SITES

Any attempt to understand the effects of chemicals on bivalve defense capacity must confront the conceptual obstacles noted at the beginning of Section II; the types and concentrations of chemicals vary in the water column over time and each chemical exhibits a unique rate of assimilation and elimination. Furthermore, variability in the natural environment can influence amebocyte defense responses (Fisher 1988). Oysters are poikilothermic and osmoconforming, so the frequent fluctuations in temperature and salinity of estuarine and coastal waters are reflected in oyster tissues. Temperature and salinity are both known to affect defense-related activities of eastern oyster amebocytes (Fisher & Newell 1986, Fisher & Tamplin 1988), as is the seasonal reproductive cycle (Fisher et al. 1989, Fisher et al. 1996).

Bivalves do not produce antibodies for specific recognition of antigens, but rely on robust, relatively non-specific cellular and humoral mechanisms to heal wounds and ward off microbial invaders. Bivalve amebocytes, also called coelomocytes, hemocytes, leukocytes, phagocytes, and blood-cells, are considered key to internal defense because of their ability to phagocytose, encapsulate, and degrade foreign material, including parasites and pathogens (Takatsuki 1934, Wagge 1955, Bang 1973, Narain 1973, Cheng 1975, 1981, 1984, Fisher 1986, Feng 1988, Chu 1988, Chu 2000). Essential to these activities is the mobility of amebocytes and their capacity to migrate across epithelial barriers (diapedesis). Defense is not the only role of bivalve amebocytes; they are reported to participate in a variety of other important biologic functions, most notably food digestion, excretion, and shell deposition (Yonge 1928, Wagge 1955, Narain 1973, Cheng 1977, Feng et al. 1977). Each of these roles is undoubtedly affected by both natural and anthropogenic stimuli in the environment.

Field Studies Positively Link Chemical Contaminants and Amebocyte Activities

Recent investigations in Florida characterized the prevailing responses of oyster defenses, particularly amebocyte-based de-

fenses, to a spectrum of pollution types and intensities under natural conditions. The approach was intended to provide a "real-world" characterization of multiple factors without regard to independent effects of individual chemicals. Eastern oysters were collected from polluted and unpolluted sites to compare tissue chemical residues and defense activities. Although immunosuppression is a common expectation, the studies revealed a higher level of amebocyte activities for oysters collected at the more polluted sites. In the first study (Fisher et al. 2000), oysters were collected from 16 heavily-, moderately-, and lightly-contaminated sites in Tampa Bay (Long et al. 1991, Long et al. 1994, McCain et al. 1996). Circulating amebocytes of oysters at the more contaminated sites exhibited higher numbers and higher locomotory activity (Table 9). Positive associations with these putative defense characteristics were strongest at sites where oysters had high concentrations of metals, particularly copper and zinc, and polycyclic aromatic hydrocarbons (PAH). Based on a preconception that metals were sequestered solely for detoxification and elimination, it was suggested that the presence of high ambient metal concentrations triggered an elevated amebocyte response to protect the organism. Enhancement of defense activities was believed incidental to detoxification (Fisher et al. 2000).

A subsequent survey (Oliver et al. 2001) explored the geographic extent of this association by examining oysters collected from 22 locations across 5 Florida bays (St. Andrew, Choctawhatchee, Pensacola, Tampa, and Biscayne Bays, Fig. 13). Chemical concentrations and defense factors varied across bays and among sites within a bay. Within-bay comparisons reiterated the finding that oysters inhabiting contaminated sites had higher numbers of circulating amebocytes, higher percentages of mobile amebocytes, and higher rates of locomotion. These characteristics were positively associated with site-averaged concentrations of metals, particularly copper, tin, and zinc (Table 10), PAHs, and even with certain polychlorinated biphenyls (PCB). Data analyzed across all five bays also revealed a strong and consistently positive association between tissue chemical concentrations and amebocyte measurements. These results, corroborating those of the first study, led to a projection that the prevailing effect of environmental mixtures of chemicals on defenses of Florida oysters was enhancement,

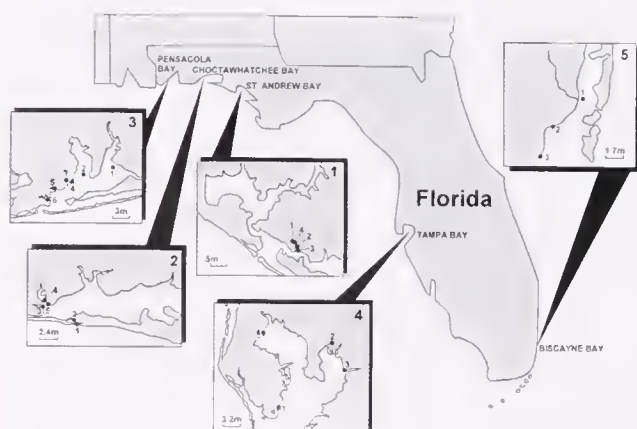


Figure 13. Oyster collection sites (22) from five bays across Florida. Chemicals analyzed from oyster tissues included metals, PAHs, PCBs and pesticides. Measurements on oyster amebocytes included density (number mL^{-1} hemolymph), percent locomotory and rate of locomotion (Oliver et al. 2001).

rather than suppression, of amebocyte numbers and defense capabilities.

Technical Constraints and Resolutions

Understanding the prevailing effect of enhanced defenses at contaminated sites was limited by two technical constraints. First, measurements of different amebocyte activities in both studies were performed as indicators of defense capacity ("putative" defense characteristics), rather than direct measures. Although it is easily accepted that more, and more highly active, amebocytes should augment the defense response, clear evidence of elevated microbicidal activity would have been profoundly more convincing. Accordingly, a vertebrate procedure to estimate microbial killing by amebocytes *in vitro* was adapted (Volety et al. 1999). *Vibrio parahaemolyticus*, common bacteria in oysters and coastal environments, were incubated with constant numbers of circulating

TABLE 9.

Summary of results from oysters collected at 16 sites in Tampa Bay, Florida (Fisher et al. 2000). *Top*: Number of significant positive and negative associations ($P < 0.05$) between amebocyte defense characteristics and various chemicals analyzed from oyster tissues. Defense characteristics include amebocyte density (number mL^{-1} hemolymph), percent locomotory amebocytes, and rate of amebocyte locomotion. Associations were determined by comparison of pooled chemical data from each site (20 oysters) with averages of 20 individuals from the same site. *Bottom*: R-values for copper, tin, and zinc, all exhibiting significant positive associations.

Association	Amebocyte Density	Percent Locomotory	Rate of Locomotion
Positive	24	26	10
Negative	2	1	2
Metals			
Cu	0.748	—	—
Sn	0.622	—	0.565
Zn	0.605	—	0.546

TABLE 10.

Summary of results from oysters collected at 22 sites across five Florida bays (Oliver et al. 2001). *Top*: Number of significant positive and negative associations ($P < 0.05$) between amebocyte defense characteristics and chemicals analyzed from oyster tissues. Defense characteristics include amebocyte density (number mL^{-1} hemolymph), percent locomotory amebocytes, and rate of amebocyte locomotion. Associations were determined by comparison of pooled chemical data from each site (20 oysters) with averages of 20 individuals from the same site. *Bottom*: R-values for arsenic, copper, tin and zinc, all exhibiting significant positive associations.

Association	Amebocyte Density	Percent Locomotory	Rate of Locomotion
Positive	25	26	4
Negative	0	0	2
Metals			
As	—	0.538	—
Cu	—	0.499	0.570
Sn	0.665	0.464	0.415
Zn	0.463	—	—

amebocytes. After a challenge period, numbers of surviving bacteria were estimated by a colorimetric assay based on their reduction of tetrazolium dye. This technique, applied in this and other studies (Genthner et al. 1999, Volety & Fisher 2000), provided a direct and more definitive assessment of eastern oyster defense capacity.

The second technical constraint involved the decision to analyze chemical concentrations from a single composite of 20 oysters collected from each site, an experimental compromise necessitated by the need for sufficient tissue to assay a broad array of chemicals. This decision negated the statistical correlation of specific analytes with specific amebocyte characteristics, which were measured independently for each oyster. Chemical concentrations in oysters at the same site are known to be highly variable (see Section II), and a single composite value could not adequately reflect concentrations of an individual.

Deployment Studies in Pensacola Bay Area

To overcome these two technical constraints, a subsequent study measured metals from individual organisms and applied the amebocyte killing assay as a direct estimate of amebocyte defense activity (Oliver et al. 2003). Only two sites in Pensacola Bay were sampled, but they differed markedly in type and magnitude of chemicals present. Chemical analyses were confined to metals and butyltins from individual oysters at each site, which allowed statistical correlation with amebocyte measures from the same oyster. Additional chemicals were analyzed as previously, from a composite. Oysters from one site (Bayou Chico) bore significantly higher concentrations of Cu, Mn, Sn, Zn, butyltins, PAH, and PCB and displayed significantly higher circulating amebocyte numbers and bactericidal activity. These oysters exhibited significantly lower concentrations of Al, Cr, Fe, Ag, Cd, and Hg than oysters from the East Bay site. Statistically-significant correlations were found between defense measurements and specific analytes (Table 11). Corroborating the previous findings, circulating amebocyte numbers and bactericidal activity were positively correlated with Cu, Sn, Zn, total metals, tributyltin, and total PAH.

Although an association between chemicals and defense activities was reasonably established, it was still unknown whether elevated defenses were induced through short-term chemical exposure (acclimation), were selected through survival of defensively-active oysters at chronically contaminated sites (adaptation), or both. An additional study was performed to determine whether short-term exposures would alter amebocyte activity (Fisher et al. 2003). Hatchery-reared oysters were deployed for 12 weeks in the summer at 3 sites in Pensacola Bay, Florida. The sites (Bayou Chico, Bayou Texar, and Santa Rosa Sound, respectively) were heavily-, moderately- and lightly-influenced by anthropogenic discharges. Tissue concentrations of Cu and Zn (Fig. 14) as well as Cr, butyltins, and PAH increased dramatically at Bayou Chico during deployment. Concomitantly, amebocyte number and bactericidal activity were significantly elevated at Bayou Chico. These results, while not excluding adaptation, demonstrated that amebocyte enhancement could be an acclimation response. Positive associations of chemicals with amebocyte number and activity corroborated the earlier studies, but results from a 16-week spring deployment were equivocal, implying that the stimulus, response, or capacity to detect a response was seasonally-dependent or influenced by other environmental factors.

Results from these field studies consistently demonstrated that

TABLE 11.

Correlation analysis between amebocyte characteristics and tissue metal concentrations in individual oysters analyzed by Oliver et al. (2003). Twenty oysters were analyzed from each of two sites ($n = 40$) differing dramatically in chemical contamination. Bacterial killing index is the percent of bacteria (*Vibrio parahaemolyticus*) killed *in vitro* and amebocyte density is the number of amebocytes mL^{-1} hemolymph. Pearson's correlation coefficients are reported as significant at $P < 0.05^*$, significant at $P < 0.01^{**}$, or not significant (ns). Positive correlation coefficients are shown in bold type.

Metal	Bacterial Killing Index	Amebocyte Density
Ag	-0.689**	-0.437**
Al	-0.487**	ns
Ba	-0.387*	-0.323*
Cd	-6.18**	-0.381*
Cr	-0.571*8	-320**
Cu	+0.672**	+0.462**
Fe	-0.543**	ns
Hg	-0.702**	-0.441**
Mn	+0.364*	ns
Pb	ns	ns
Sb	-0.468**	ns
Sn	+0.702**	+0.485**
Zn	+0.738**	+0.488*
TBT	ns	ns
DBT	+0.682**	+0.614**
All Metals	+0.731**	+0.490**

TBT, tributyltin; DBT, Dibutyltin.

All Metals represents a composite analysis of all metals measured.

environmental contaminants stimulated, rather than suppressed, amebocyte defense activities. The results portrayed cumulative effects of multiple chemicals under natural environmental conditions, not the effects of individual chemicals that might independently suppress or stimulate amebocytes. The studies did not examine, despite the obvious implication, any mechanistic linkage to oyster disease resistance. However, they established a consistent and defensible relationship between amebocyte numbers and defense activities with tissue concentrations of copper and zinc. These were not the only two analytes associated with elevated defenses but, because of their dominating concentrations in oysters and their physical containment within amebocytes, a possible cause-effect relationship became worthy of consideration.

ANTIMICROBIAL FUNCTIONS OF COPPER AND ZINC

It was reported in Section III that accumulations of copper and zinc in oysters were so extraordinarily high that many investigators believed they exceeded useful amounts (Hiltner & Wichmann 1919, Bodansky 1920, Korrington 1952, Pequegnat et al. 1969, Wolfe 1970a, Simkiss & Mason 1983).

"It seems probable that zinc, as well as copper, can be absorbed and retained in the tissues of the oysters in quantities far in excess of functional requirements, especially in oysters grown in waters badly polluted with metallurgical and factory wastes." (Hiltner & Wichmann 1919, p. 221)

Functions that might be served (e.g., respiratory pigment) (Mendel & Bradley 1905, Prytherch 1934) or enzyme catalysts (Pequegnat et al. 1969), simply did not require the high concentrations accumulated in tissues. Ruddell and Rains (1975) hypothesized that the metals might be used in numerous oyster functions.

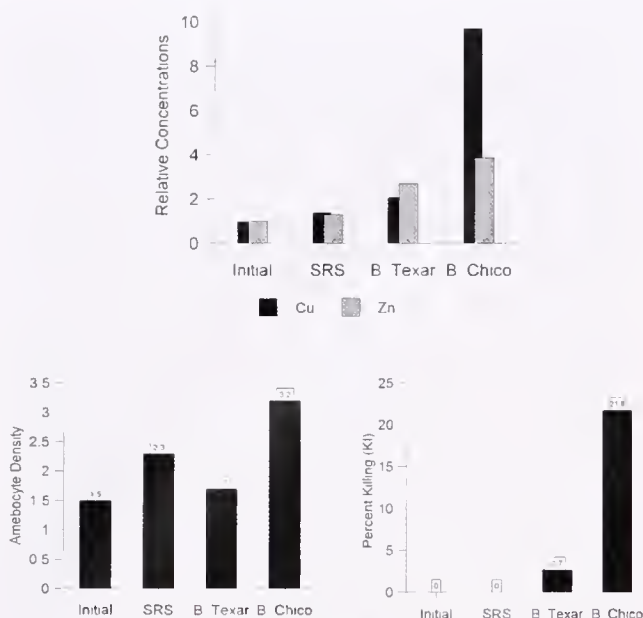


Figure 14. Comparison of hatchery oysters at the time of deployment (initial) and after 12 weeks at Bayou Texar, Bayou Chico, and Santa Rosa Sound (SRS), all sites in the Pensacola Bay area (Fisher et al. 2003). Top: Copper and zinc concentrations in oyster tissues normalized to the initial concentrations ($46 \mu\text{g copper g}^{-1}$ and $1230 \mu\text{g zinc g}^{-1}$) show elevated concentrations at Bayou Chico. Bottom left: Amebocyte densities ($\times 10^6 \text{ mL}^{-1}$ hemolymph) were significantly elevated at Bayou Chico over initial values. Bottom right: Ability to kill bacteria (*Vibrio parahaemolyticus*) under *in vitro* conditions was elevated in amebocytes from Bayou Chico. Killing index is percent of bacteria killed by a constant number of amebocytes.

including hydration of CO_2 , maintenance of extracellular and intracellular pH, production or entrapment of free radicals, regulation of redox potentials, or traps for small molecular weight compounds with affinities for zinc or copper. Yet, these roles would be difficult to perform for metals sequestered within amebocyte granules and, even if true, all these functions combined would probably not require the concentrations that can be accumulated.

This review rekindles a premise of functional roles for high accumulations of copper and zinc. Field studies described in Section IV demonstrated greater amebocyte numbers, mobility and bactericidal activity in eastern oysters inhabiting sites contaminated with high levels of copper and zinc. In particular, elevation of bactericidal activity implicated a true physiologic function for copper and zinc in oyster defense. Such a role, in fact, had already been proposed, as described below.

Copper and Zinc in Wound Healing

Ruddell (1971) confirmed the observations of Pauley and Sparks (1965) and DesVoigne and Sparks (1968) that green coloration often accompanied the healing of wounds in *C. gigas*. This coloration Ruddell interpreted as an inflammatory response involving copper-laden amebocytes, an association that had been well established (Section I). In his experiments, Ruddell provoked an inflammatory response by wounding the mantle tissues or implanting excised oyster tissue into an intact mantle cavity. Tracking the tissue changes histologically, he found that copper did, indeed, appear at the site of the wound, first bound within the granules of acidophilic granular amebocytes (AGA), but later in

extracellular spaces bound to surfaces of muscle, nerve and epithelial tissues near the wound. Copper was subsequently found in granules of basophilic granular amebocytes (BGA) at the wound site, but not in BGAs distant from the wound site. These observations he interpreted to mean that copper, which was accumulated primarily in AGAs, was released at the site of a wound, distributed to tissues near the wound area, and then recovered into granules of BGAs. From these observations, Ruddell (1971) proposed that copper served as an antimicrobial agent:

"Although it is apparent that copper plays an essential role in the oyster inflammatory response, the function of the copper in the response is not known. One can presume that copper might function to ward off, destroy, or inhibit the growth of potential oyster pathogens." (Ruddell 1971, p. 110)

Ruddell (1971) also hypothesized a similar role for zinc. His evidence indicated that BGAs recruited to the site of a wound underwent swelling and released zinc from their granules. He also noted the release of other constituents, in particular diazo-positive material that he believed was a phenolic substance.

Using both *C. gigas* and *C. virginica*, Ruddell and Rains (1975) formulated a functional role for high accumulations of copper and zinc. Although they understood that zinc- and copper-rich amebocytes could be used in diverse ways, they believed that a primary role was in response to trauma.

"It would seem probable that oysters do use their basophils and hence, by extension, the large amounts of zinc and copper incorporated in the basophils. Therefore, although oysters may contain very large quantities of zinc and copper, these metals must not be regarded as being stored away in a physiologically inactive form." (Ruddell & Rains, p. 590)

Their studies provided compelling rationale for extracellular killing by copper and zinc, serving in their well-known capacity as "antimetabolites for a diverse number of animal, plant and microbial forms." Direct evidence of microbial killing is lacking, however, even though concentrations of copper and zinc in amebocytes (see Table 6) seem more than adequate to effect toxic action.

Extracellular Clot Formation

Ruddell's demonstration of extracellular release of copper and zinc led to later research on the formation of extracellular clots. Most organisms have some mechanism to form clots in the hemolymph or blood; clotting is a sequence of complex chemical and physical reactions that results in conversion of fluid (hemolymph or blood plasma) into a coagulum. Clotting in invertebrates, as described by Gregoire (1970), involves the exudation or eruption of blood cells to release granular material into the surrounding fluid. Circular clouds of a granular consistency form around the blood cells, and they eventually develop into "islands of coagulation" formed by networks of granular fibrils. In most early studies, lamellibranch hemolymph was found to lack extracellular clotting. Takatsuki (1934) noted clumping of amebocytes withdrawn from *O. edulis*, but he did not observe any extracellular clot formation.

"The amebocytes of the oyster were allowed to stand after being drawn from the body: the mass of amebocytes did not become jelly-like by coagulation as in the case of crustacean and vertebrate blood, and no fibrin appears in the blood-plasma which remains fluid on standing in the air and even after being heated." (Takatsuki 1934, p. 402)

Eastern oysters became the first, and may well be the only, bivalve known to exhibit such a response (Narain 1973). Frederick B. Bang (1961) described clotting in eastern oysters as an extracellular, finely-granular "gel" that occurred around aggregates (clumps) of amoebocytes (Fig. 15). Clots were observable on glass slides under phase-contrast microscopy within 30 min of hemolymph withdrawal, and they resembled clots formed naturally in the hemolymph vessels of traumatized oysters. He noted that these clots could trap and immobilize bacteria, presumably making the bacteria more susceptible to phagocytosis.

"It seems to have a real rôle in the repair of traumatized tissue, for: 1) it was found already developing in small cellular clots taken directly from the heart; 2) it occurred predominantly around clumps of cells; 3) bacteria were immobilized by its development . . . and, 4) it was obtained both immediately after opening an oyster and from some preparations which had been on the half shell as long as 24 h. Its possible relation to cycles of feeding by the amoebocytes is unknown." (Bang 1961, p. 61)

Copper and Zinc Initiate Extracellular Clot Formation

Bang (1961) felt that the extracellular gel in eastern oysters may have originated from extrusions of the cellular granules of the amoebocytes (degranulation), a process previously described for arthropods (Gregoire 1970). It was not until a decade later that one of Bang's doctoral students, Robert S. Brown, followed up on this hypothesis. He noted that extracellular clots would sometimes

form when whole hemolymph or plasma was added to glass slides treated with alconox detergent (R. S. Brown, personal communication). Subsequently, he found that other detergents and a variety of other substances, including bases, alcohols, copper, and zinc, would also cause extracellular clotting (Fig. 16). Recognizing that copper and zinc were used in biochemical separation methods to precipitate proteins, Brown combined the work of Bang (1961) and Ruddell (1971) to forge his dissertation hypothesis that copper and zinc released from amoebocyte granules precipitated hemolymph plasma proteins to form extracellular clots (Brown 1975). To test his hypothesis, Brown investigated 3 basic suppositions; (1) that oyster amoebocytes were the source of the clot-promoting substance, (2) that copper and zinc were released from amoebocytes into the plasma, and (3) that copper and zinc produced clots in plasma by precipitating proteins.

Using a variety of microscopic and analytical tools, Brown (1975) was able to support each of the suppositions. He reiterated Bang's finding that extracellular clots were always associated with

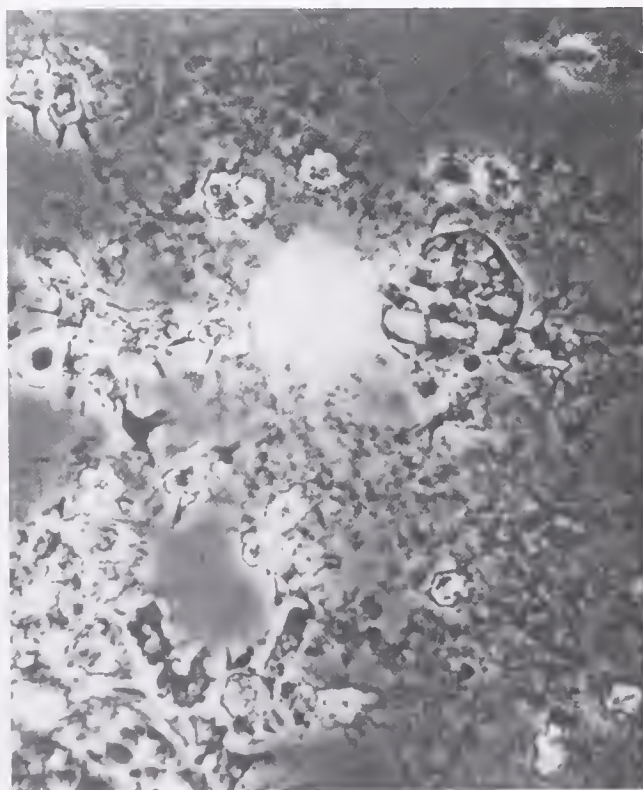


Figure 15. Eastern oyster amoebocytes and extracellular clotting of hemolymph shown as coarse, granular areas (phase-contrast microscopy, $\times 1,664$). Reproduced from a duplicate published in Brown (1975; Fig. 11 with author's permission) of the first published micrograph of extracellular clotting in bivalves by Frederick B. Bang (1961; Fig. 6, with permission of the *Biological Bulletin*).

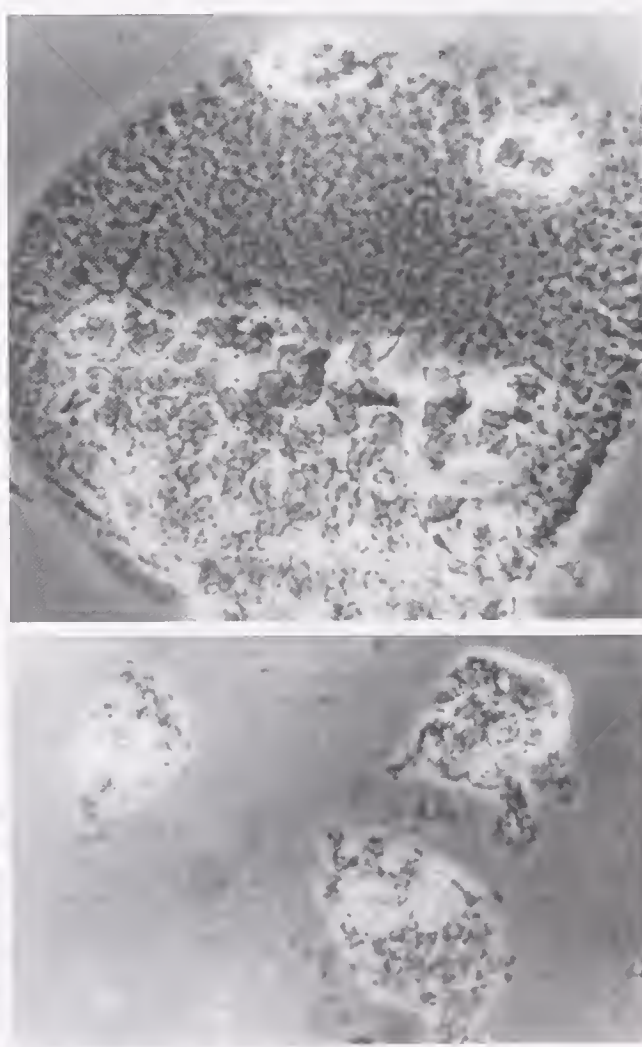


Figure 16. Eastern oyster amoebocyte aggregates with extracellular clots forming a network around the cells ($\times 480$). Top: Clot formed immediately after addition of 0.1M NaOH to whole hemolymph. Bottom: Clots formed immediately after addition of 0.5% detergent to whole hemolymph. (Reproduced from Brown 1975; Fig. 17 with permission of the author).

amebocyte aggregates (Fig. 17) and would not form in hemolymph if amebocytes were removed. Clots would form if amebocytes were added back to cell-free hemolymph, even if the amebocytes had been fixed with glutaraldehyde, or were frozen, boiled and extracted before reintroduction. He verified for *C. virginica* the earlier evidence (Ruddell 1971) that *C. gigas* amebocytes sequestered copper and zinc (see Figs. 6 and 7) and that they were retained in amebocyte granules (see Figs. 8 and 9). He used a combination of techniques to verify that high concentrations of copper and zinc were excluded from agranular regions of the amebocytes (See Fig. 10).

To provide some evidence that amebocytes could release copper and zinc from the granules, Brown (1975) presented electron microscopic evidence that independent amebocytes were highly granulated, whereas those amebocytes in aggregates were either completely degranulated, in the process of degranulation, or in some cases, undergoing cytolysis (Fig. 18). He believed degranulation provided a mechanism for release of copper and zinc into the hemolymph. He also revealed that extracellular clots had a relatively high staining affinity (Fig. 19) and high concentrations of copper ($356 \mu\text{g g}^{-1}$, dry weight) and zinc ($10,571 \mu\text{g g}^{-1}$); these were substantial amounts, particularly in comparison to the minute quantities found in clot-free plasma (copper $< 0.2 \mu\text{g mL}^{-1}$ and zinc $< 10 \mu\text{g mL}^{-1}$).

Finally, Brown (1975) demonstrated that addition of either Cu-acetate or Zn-acetate to cell-free oyster plasma created precipitates that were indistinguishable from *in vitro* clotting (Fig. 20). Precipitation required a minimum of either $0.6 \mu\text{g Cu mL}^{-1}$ or $0.15 \mu\text{g Zn mL}^{-1}$ hemolymph and, as expected, precipitation dramatically reduced the concentration of plasma proteins. When plasma was first deproteinized with trichloroacetic acid, no amount of copper or zinc would cause precipitation. Similarly, if copper were added to zinc-treated plasma, no further precipitation resulted. Boiling of the plasma did not influence the ability of copper or zinc to effect precipitation, leading to Brown's conviction that extracellular clot formation was a non-enzymatic chemical action. He found that ethylenediaminetetra-acetic acid (EDTA) and hydro-

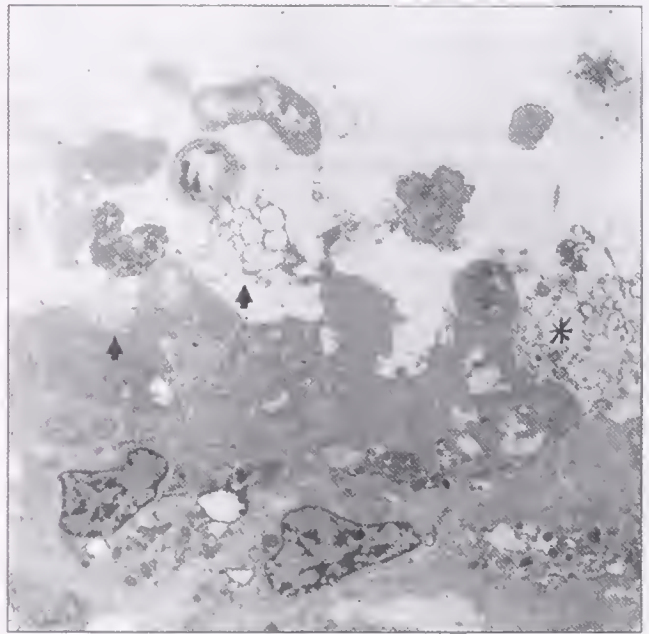


Figure 18. Transmission electron micrograph of a peripheral edge of aggregated eastern oyster amebocytes ($\times 10,000$). Although not visible in this preparation, an extracellular clot was formed from this aggregate. Amebocytes appear agranular or in the process of degranulation (arrows). Some cells appear to be lysed (asterisk). (Reproduced from Brown 1975; Fig. 14 with permission of the author).

chloric acid (HCl), both known to disperse metal-protein precipitates, also dispersed extracellular clots.

In all, Brown's (1975) data indicated that copper and zinc from amebocytes could interact with proteins in the hemolymph *via* a non-enzymatic, inorganic reaction to form locally-restricted precipitations, or clots, consisting of calcium and magnesium hydroxide, phosphates and carbonates. There may be other constituents released simultaneously by the amebocytes, such as the phenols described by Ruddell (1971), that may also play a role in clot formation. Phenoloxidase is a constituent of the prophenoloxidase clotting system in crustaceans (Söderhäll 1982, Söderhäll et al. 1994) that is believed to provide melanin for encapsulation of invading microorganisms. Although not linked to a clotting mechanism, phenoloxidase activity has also been reported in bivalves (Coles & Pipe 1994, Asokan et al. 1997, Carballal et al. 1997, Deaton & Dankert 1998, Peters & Raftos 2002).

Brown (1975) did not analyze the form of copper and zinc in the granules of amebocytes or in the extracellular clots. The work of Coombs (1972) led George et al. (1978) to conclude that granules contained copper sulfide and zinc phosphate. Metal speciation may be important because it could control the pH of material released from the amebocyte granules. Either high or low pH could contribute to release of metals from the granules and protein precipitation in the hemolymph. Beaven and Paynter (1999) have shown that phagosomes (see below) become highly acidified after fusion with lysosomal granules.

Intracellular Microbicidal Potential of Copper and Zinc

Ruddell & Rains (1975) confined their speculations to the extracellular microbicidal capacity of copper and zinc in wound healing. However, the possibility that these metals participate in intracellular killing by amebocytes is equally compelling. Metchnikoff

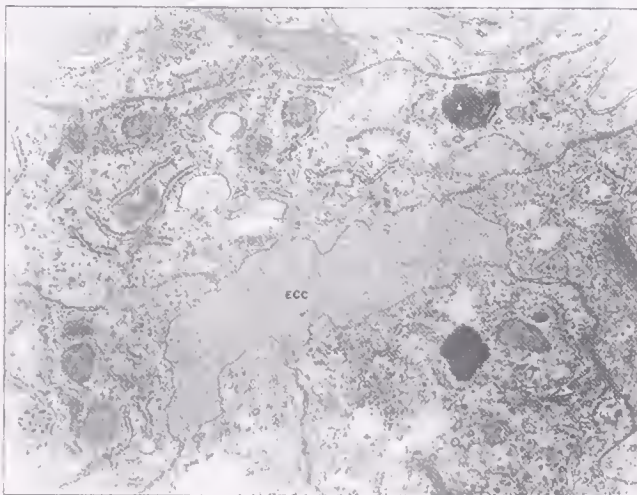


Figure 17. Electron micrograph ($\times 23,500$) of an extracellular clot (ECC), which appears as a finely granular material between the junction of five aggregated amebocytes taken from a mantle wound of an eastern oyster. (Reproduced from Brown 1975; Fig. 13 with permission of the author).

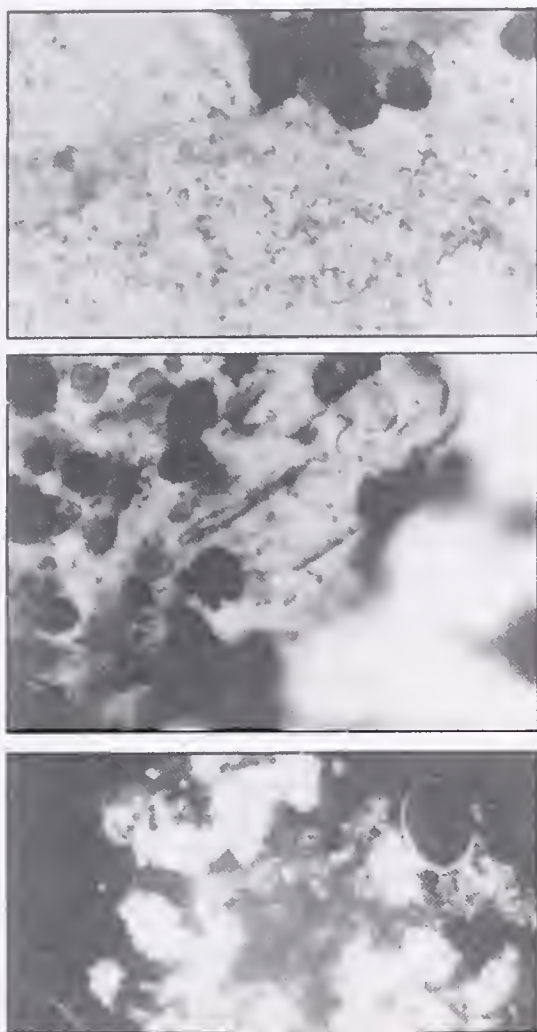


Figure 19. Histochemical demonstration of copper and zinc in extracellular clots of eastern oyster plasma. Top: Extracellular clot formed *in vivo* showing positive reaction for copper when stained with Mallory's hematoxylin stain ($\sim 500\times$). Middle: Different field from the same clot as above (higher magnification). Bottom: Fluorescent yellow staining of zinc with 8-hydroxyquinoline stain on an *in vitro* extracellular clot ($\times 150$). (Reproduced from Brown 1975; Fig. 33 with permission of the author).

(1891) brought attention to evidence that vertebrate and invertebrate amoebocytes possess phagocytic ability. A complex process, phagocytosis requires that amoebocytes recognize, locate, ingest, and either transport or digest foreign particles. In eastern oysters, as in other bivalves, phagocytosis is considered critical to both nutrition and internal defense (Cheng 1977, Feng et al. 1977). To provide nutrient, amoebocytes migrate into the digestive tract to engulf particles of food, digest them, and then migrate into the tissues and hemolymph to release nutrient (usually as glycogen) to metabolizing cells. Yonge (1937, 1946), among others, believed this to be the primary nutritional pathway for oysters. Using a similar process, amoebocytes can capture unwanted particles and microorganisms (parasites and pathogens) in the mucus and hemolymph to prevent microbial growth in the soft tissues. If the phagocytosed material has no nutritive value, it can be eliminated through feces or pseudofeces by amoebocyte exomigration (see

PRECIPITATION OF OYSTER PLASMA PROTEIN BY COPPER AND ZINC

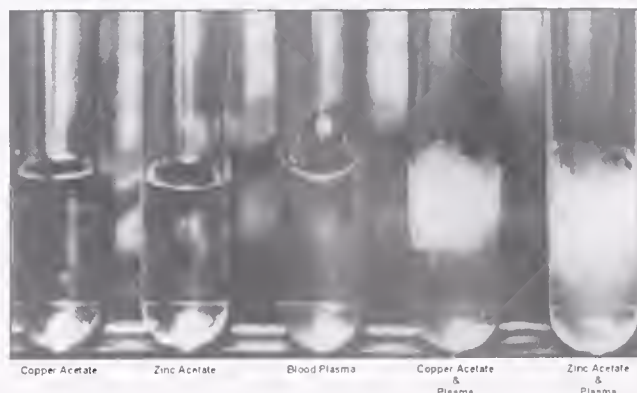


Figure 20. Demonstration by Brown (1975) of precipitation of eastern oyster hemolymph plasma by copper and zinc. A clot-like precipitate formed in cell-free plasma immediately after addition of copper or zinc, whereas no precipitate formed if either the plasma or the metal was absent. Precipitation of plasma by one agent eliminated further precipitation by later addition of other agents. (Reproduced from Brown 1975; Fig. 31 with permission of the author).

Section II). It has been postulated that phagocytosis was originally a nutrient-acquiring process that eventually evolved into a defensive capability (Cheng 1975, Feng et al. 1977).

Professor Leslie A. Stauber (1950) initiated the first of many studies that characterized phagocytosis in marine bivalves when he traced the fate and disposition of injected india ink particles in *C. virginica*. Three investigators, Sung Y. Feng, Marennes R. Tripp and Thomas C. Cheng, followed Stauber's initiative with innumerable experiments and cogent descriptions of bivalve internal defense, often using eastern oysters as a model system (see Cheng 1983). Iterative updates on their progress were provided by Cheng (e.g., Cheng 1967, 1975, 1981, 1984, 1996). Combined, these studies have detailed the fates of a diverse array of foreign particles and microorganisms in oysters and characterized the process of phagocytosis (e.g., Stauber 1950, Stauber 1961, S. Y. Feng 1958, 1966, 1967, 1988, Tripp 1958, 1960, 1970, J. S. Feng 1966, Tripp & Kent 1967, Acton et al. 1969, Fries & Tripp 1970, Feng et al. 1971, 1977, Cheng & Cali 1974, Foley & Cheng 1975, 1977, Cheng & Rudo 1976, Renwranz et al. 1979, Hinsch & Hunte 1990). Most living microorganisms are killed upon ingestion by phagocytic amoebocytes, including viral particles (J. S. Feng 1966, Fries & Tripp 1970) bacteria, phytoplankton (e.g., *Navicula ostrearia*), and metazoans. But some microorganisms are able to survive and even multiply within the phagosomes. Of particular interest for eastern oysters is the apparent ability of *Perkinsus marinus*, a devastating oyster pathogen, to grow and multiply within host amoebocytes (Mackin 1951).

Ingestion, or endocytosis, of microorganisms by eastern oyster amoebocytes reportedly can occur in three ways; some microbes adhere to the amoebocyte filopodia (Bang 1961), some are taken into funnel-shaped pseudopods (Renwranz et al. 1979), and some are enveloped in a vesicle on the amoebocyte surface (Cheng 1975). Regardless of the path of entry, the targeted microorganism is captured and drawn into a cytoplasmic invagination (Fig. 21) that encircles it with concentric lamellae to form a "phagosome" (Cheng 1975). Digestion of the microbe begins in this primary phagosome but, at least for eastern oysters, can be transferred to, or evolve into a secondary phagosome which has an electron-dense

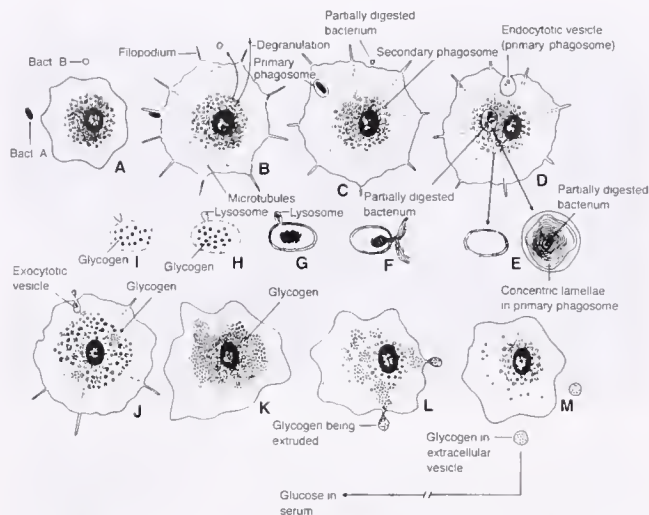


Figure 21. Description of phagocytosis of bacteria by eastern oyster amebocytes as described by Cheng (1975). Granules participate in both intracellular and extracellular degradation. Intracellular digestion occurs after a bacterium is isolated in a phagosome, then exposed to degrading enzymes released when the phagosome fuses with cytoplasmic granules (lysosomes). This can result in the eventual release of glycogen as a nutritional product. Extracellular degradation of bacteria occurs when amebocytes "degranulate" or release the hydrolytic enzymes of the lysosomes outside the cell. (Diagram from Cheng 1996; Fig. 11 with permission of Maryland Sea Grant).

outer surface comprised of a thick outer wall. Lysosomes, which are membrane-lined organelles containing acid hydrolases, have been observed to fuse with secondary phagosomes (Cheng 1975), and may fuse with primary phagosomes as well (Cheng 1996). Fusion of lysosomes with phagosomes is believed to expose the microorganism to degrading lysosomal enzymes. The fused organelle is sometimes called a "phagolysosome". Fusion with a lysosome results in relatively rapid acidification of the phagosome (Beaven & Paynter 1999).

Both granular and agranular (hyaline) amebocytes have been found capable of phagocytosis, but granular forms are recognized to be much more active. Foley and Cheng (1975) found over 80% of the granular amebocytes in *C. virginica* to phagocytose *Staphylococcus aureus* and *Escherichia coli* compared with less than 20% of hyaline amebocytes. With the exception of Ruddell (1971), who may not have included degranulated cells (fibrocytes) in his estimates (see Cheng 1981), it is generally maintained that granular amebocytes are more abundant and more actively phagocytic in oysters than agranular amebocytes. This may be because granules play a critical role in the phagocytic process. In fact, granularity and phagocytic activity may be tightly linked; Beaven and Paynter (1999) discovered that the number of visible granules in eastern oyster amebocytes declined as phagocytosis proceeded. It could be inferred that highly granular amebocytes are avidly phagocytic, but lose their potential as more granules fuse with phagosomes.

The amebocyte granules that contain copper and zinc are morphologically indistinguishable from lysosomes, and there is little reason to believe that they are not the same. Ruddell (1971) called the copper and zinc granules "lysosomal derivatives", but did not elaborate on similarities or differences. From the results of Brown (1975), it seems that virtually every granule in the eastern oyster amebocyte contains some copper or zinc (see Figures 6–9). There

has been some suggestion that granules and lysosomes in eastern oysters differ morphologically (Feng et al. 1971, Cheng 1975), but distinctions between the two are vague. Yoshino and Cheng (1976) demonstrated through cytochemistry and electron microscopy that the granules in amebocytes of *Mercuraria mercenaria* were lysosomes, serving as storage organelles for hydrolytic acids. Foley and Cheng (1977) showed that lysosomes can be stimulated to move to the surface of the amebocyte and expel their contents (Fig. 22), a process that resembles, or is identical to the degranulation process described by both Ruddell (1971) and Brown (1975) for metal-containing amebocyte granules. It can be postulated from these observations that the copper- and zinc-bearing granules in eastern oysters are either lysosomes or closely-related structures.

It is possible to hypothesize a role for copper and zinc in intracellular microbicidal activity. When phagosomes fuse with cytoplasmic granules, the captured microorganisms are exposed to very high, and likely toxic, concentrations of metals and hydrolytic enzymes. Unlike the case for extracellular killing, metals used in intracellular killing remain inside the cytoplasm. If the phagosome breaks down, as has been described for the release of glycogen granules in nutritive digestion, the metals could be recovered by newly-formed granules.

Extracellular Toxicity to Encapsulated Microorganisms

A similar extrapolation might be drawn for the role of copper and zinc in amebocyte action against larger microorganisms (i.e., parasites that have been encapsulated). Encapsulation is the simultaneous envelopment of relatively large foreign bodies by numerous amebocytes (Fig. 23). The foreign body is usually too large for a single amebocyte to ingest; nonetheless, encapsulation may be the consequence of independent attempts by many amebocytes to phagocytose the invader (Cheng & Rifkin 1970). The cells become flattened against the surface of the foreign body and ultimately cut it off from host tissues.

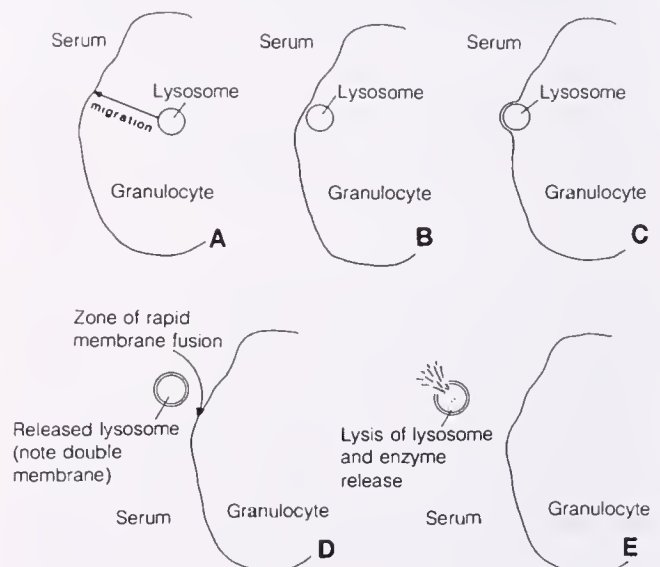


Figure 22. Schematic diagrams of degranulation, or release of lysosomes from a bivalve granulocyte. (A–C) Lysosomes migrate to and protrude through the plasma membrane. (D) Double-membraned lysosomes are exocytosed from the granulocyte and eventually lyse (E) to release lysosomal enzymes. It is likely that the same mechanism releases copper and zinc from amebocyte granules. (Figure from Cheng 1996, Fig. 15 with permission of Maryland Sea Grant).

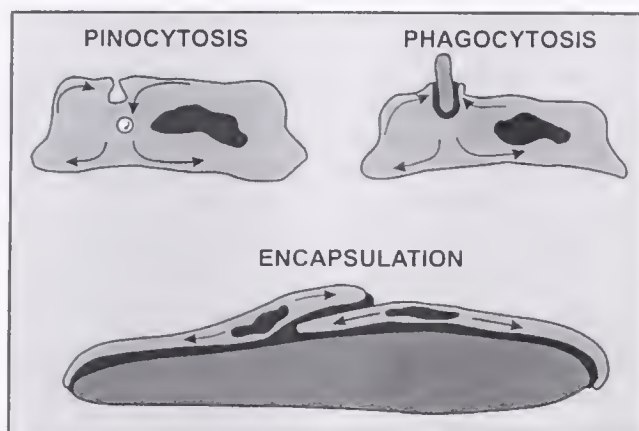


Figure 23. Amebocytes can respond to foreign objects in three ways: (1) *pinocytosis*, or cell-drinking, is a means to uptake small or dissolved particles and fluid; (2) *phagocytosis*, or cell-eating, is a means to take up larger particles; (3) *encapsulation* occurs when the foreign object is too large for a single amebocyte to phagocytose. Bang (1973) suggested that pinocytosis involved ingestion without recognition, encapsulation involved recognition without ingestion, and phagocytosis involved both recognition and ingestion. (Graphic redrawn from Bang 1973, Fig. 2).

It is not unlikely, assuming that encapsulation is an attempt at phagocytosis, that encapsulating amebocytes degranulate while encompassing the intruder. As noted by Cheng (1981),

"... external contact with certain foreign substances will result in hypersynthesis of intracellular lysosomal enzymes which are released from haemocytes into the serum where digestion of the foreign material, such as bacteria, is initiated. The release of enzymes is effected by what has been termed degranulation (Foley and Cheng, 1977), a process involving the migration of lysosomes to the surface of the cell where the enclosed enzymes are discharged." (Cheng 1981, p. 286)

Cheng et al. (1975) demonstrated the extracellular release of lysozyme from lysosomes during phagocytosis by *Mercenaria mercenaria* amebocytes. Similarly, McDade and Tripp (1967) demonstrated the presence of lysozyme in hemolymph of eastern oysters. Extracellular release of copper and zinc at the site of a wound (Ruddell 1971) is presumably a consequence of amebocyte degranulation, as is copper-plating of a "well-polished steel knife" (Galtsoff 1964, p. 388). If metals and hydrolytic enzymes are expelled onto the surface of the invader, then the process of encapsulation attains a dual objective of both isolating and debilitating the invader.

Rationale for High Accumulations of Copper and Zinc

Evidence has been presented that copper and zinc serve antimicrobial functions in eastern oysters, yet it is not immediately obvious why such high accumulations are needed. Brown (1975), for example, estimated that the amounts of copper and zinc contained in eastern oyster amebocytes was much greater than thresholds necessary to form extracellular clots. Confounding the issue is the fact that living, healthy oysters exhibit widely varying tissue concentrations. The best explanation may simply be that higher concentrations have the capacity to generate a larger clot, or marshal stronger microbicidal action against phytoplankton, bacteria, and viruses. This increased capacity provides a competitive advantage to oysters with higher accumulated concentrations. Perhaps equally important, high accumulations may afford oysters

greater flexibility in realizing this advantage. Amebocytes are highly mobile and can move across epithelial barriers to virtually any compartment of the oyster (diapedesis). They may, however, be limited in their ability to reach a wound quickly (or an algal cell needed for nutrition) and their movement may leave other areas of the oyster unprotected (or unexploited). High accumulations of copper and zinc, combined with the requisite high numbers and high activity of amebocytes, provide the oyster a robust, distributed antimicrobial capacity that allows quick responses to frequent or repeated challenges and opportunities throughout the organism.

HYPOTHESES AND RAMIFICATIONS OF AN ALTERNATIVE FRAMEWORK

Numerous independent investigations on metals in eastern oysters have been reviewed to establish that copper and zinc, if not other terrestrial elements, are actively captured and retained for physiologic purposes. This contradicts a historical perception that high accumulations are merely part of a process to detoxify and eliminate the metals. Copper and zinc are physically associated with amebocytes, so any physiologic role is likely related to amebocyte functions. Evidence presented here links copper and zinc to antimicrobial functions of amebocytes in defense and nutrition. A separate report (Fisher 2004) presents evidence of a role for terrestrial metals in shell deposition, which is facilitated by amebocytes. The findings contradict a tendency to regard copper, zinc, and other metals as unnecessary, if not undesirable, oyster constituents. Introduced through this review is a corollary proposition that oyster success and distribution in near-coastal areas, even at seemingly polluted locations, stems from a dependency on terrestrial elements. These proposals arise from the cumulative interpretations of many scientific contributions, and generate an alternative framework for understanding issues that range from mechanisms of amebocyte function to coastal distribution of eastern oyster populations. There are numerous considerations that bear on the legitimacy of this alternative framework, some supported more than others by existing evidence, but all deserving at least some corroborative research. These considerations are listed below in a series of broad, umbrella hypotheses (H1–H6).

Eastern Oyster Adults Actively Accumulate Terrestrial Copper and Zinc

(H1) The most consistent information amassed over several years and numerous studies is that accumulation of copper and zinc is extremely high in eastern oysters, a status supported by their ability to concentrate the metals from low ambient concentrations and retain them longer than other metals despite availability of an efficient elimination mechanism. Long retention may mean that copper and zinc are transferred from old to young amebocytes by phagocytosis or apoptosis. Active assimilation and accumulation of the metals signifies a cost to oyster energetics. Accumulations of copper and zinc, rare in marine environments, represent quantities acquired from terrestrial watersheds. This factor may limit oyster distribution to estuarine and near-coastal locations.

Copper and Zinc are Sequestered in Membrane-lined Granules of Amebocytes

(H2) There can be little doubt that copper and zinc are retained primarily, if not exclusively, in oyster amebocytes. Metals have been reported in amebocytes from microscopic observations (e.g., green oysters), various histologic preparations, and electron mi-

microscopic imaging techniques. Storage in amoebocytes provides a transport mechanism to quickly distribute the metals to virtually any site in the organism. Copper and zinc are sequestered in membrane-lined granules in the amoebocyte cytoplasm at high concentrations. Sequestration shields vulnerable tissues from exposure and is the primary reason that eastern oysters can safely accumulate high tissue concentrations. Acquisition of metals by the granules probably occurs by phagocytosis of metal-laden mucus (see later) or food particles (e.g., phytoplankton and bacteria). The chemical speciation of metals in the granules allows their discharge and bioavailability upon degranulation or fusion with phagosomes.

Ambient Dissolved Copper and Zinc are Bound in Mantle-cavity Mucus

(H3) Oysters assimilate copper and zinc from ambient water or food into protective amoebocyte granules without prolonged or excessive exposure to unprotected tissues. Chemical properties of mucus ensure strong binding of any cations entering the oyster mantle cavity and prevent their absorption into tissues. Binding with mucus also provides an efficient elimination mechanism for unwanted metals; mucus accumulates in the pseudofeces, which is passed out of the body. Uptake of metals, on the other hand, may be achieved in a controlled manner by amoebocyte phagocytosis of mucus and particles bound in mucus. Phagocytic activity captures copper and zinc in membrane-lined phagosomes, vesicles formed by invaginations of the cell membrane. These vesicles may ultimately form metal-containing granules. Embryo and larval stages, without the copious mucus production of adults, are vulnerable to water-borne copper and zinc toxicity.

Copper and Zinc Accumulation Depends on Availability of Amoebocytes

(H4) Amoebocytes are highly phagocytic and can migrate throughout the alimentary tract and mantle cavity to acquire copper and zinc from food or mucus. Safe accumulation of the metals, however, depends on the availability of amoebocytes with metal-carrying capacity. Amounts of metal that can be stored in an amoebocyte are presumably constrained by its size, its capacity to form and retain granules, and the metal-carrying capacity of the granules. Whenever existing amoebocytes are saturated, additional uptake from the environment requires additional amoebocytes. Uptake by eastern oysters is thus dependent on the rate of amoebocyte proliferation or recruitment. Specific amoebocyte cell types may be recruited based on the metal present (i.e., BGA for copper and AGA for zinc). Higher numbers of amoebocytes increase the oysters' capacity to accumulate metals.

Degranulation Releases Copper and Zinc from Amoebocyte Granules

(H5) In a process diametric to endocytosis, lysosomal membranes fuse with the outer cell membrane to discharge lysozyme into extracellular spaces (degranulation). Lysosomal granules also fuse with cytoplasmic phagosomes to discharge lysozyme into phagolysosomes for killing or digesting trapped microorganisms. Amoebocyte granules that contain high concentrations of copper and zinc are similar, if not identical, to lysosomal granules. Copper and zinc, which may occur in all granules, are discharged simultaneously with lysosomal enzymes into extracellular spaces or phagolysosomes.

Copper and Zinc Provide Extracellular and Intracellular Antimicrobial Activity

(H6) Antimicrobial activities of copper and zinc include clotting of hemolymph and both intracellular and extracellular killing

of microorganisms. For intracellular killing, granules fuse with phagosomes to discharge hydrolytic enzymes and toxic concentrations of copper and zinc, which may act in concert. Toxic metals released intracellularly are excluded from vulnerable host tissues and simultaneously retained for future use. For extracellular killing, a burst of either metal during degranulation will affect invading microbes without severe damage to surrounding tissues. The high mobility and flexible morphology of amoebocytes allows degranulation to occur with relative precision at wound sites, possibly even directed at individual microorganisms. Metals are recouped to protect nearby tissues from prolonged exposure and for re-use. Extracellular discharge of copper and zinc might also provide antimicrobial activity against encapsulated microorganisms. Copper and zinc released from granules into the hemolymph precipitate proteins and generate a clot. Clotting provides a granular net to capture and immobilize bacteria and stem the flow of plasma (hemostasis). Amoebocyte clumping, or aggregation, is also a factor in hemostasis and occurs simultaneously with extracellular clot formation.

Potential Ramifications of an Alternative Framework

Anticipating that some of the considerations above are ultimately justified by direct evidence, it is worth considering potential ramifications. The most consequential aspect is the influence of copper and zinc on amoebocyte antimicrobial activity. Antimicrobial activity of amoebocytes in defense and nutritional functions has been accepted for eastern oysters, if not most bivalves, for many years (Tripp 1960, Stauber 1961, Feng 1967, 1988, Narain 1973, Anderson 1975, Cheng 1975, 1981, 1996, Fisher 1986). Some early investigators, particularly Yonge (1926, 1937, 1946), believed that amoebocytes were largely responsible for digestion of foods entering the alimentary tract. Although this emphasis has been disputed, amoebocyte participation in nutrition is widely accepted (Takatsuki 1934, Wagge 1955, Owen 1966, Purchon 1968, Narain 1973, Cheng 1975, 1977, Feng et al. 1977).

One of the greatest concerns for fisheries managers over the last 50 years has been diseases caused by 2 protozoans, *Perkinsus marinus* and *Haplosporidium nelsoni*. Host defenses against these two diseases are not well characterized, but it seems that both agents may be susceptible, at least under some conditions, to phagocytosis by oyster amoebocytes (Burrenson et al. 1988, Ford & Kanaley 1988, Sero & Ford 1990, Anderson 1996, Volety & Fisher 2000). Both diseases are severe at high salinity locations (Andrews 1988, Andrews & Ray 1988, Ford & Haskin 1988, Haskin & Andrews 1988, Ford 1996, Soniat 1996) and disease intensity can be reduced with low salinity (Sprague et al. 1969, Ford 1985, Ford & Haskin 1988, Chu & Greene 1989, Chu et al. 1993, La Peyre et al. 2003). Hence, the potential enhancement of defenses by copper, zinc, or other terrestrial elements provided by freshwater inflow could be relevant to control of disease.

Also of great concern to the oyster fishery is contamination of commercial oysters with human pathogens such as *Vibrio parahaemolyticus*, *V. vulnificus*, and *V. cholerae* (Kelly & Arcisz 1954, Blake et al. 1979, 1980, Pavia et al. 1987, Bernard 1989, Daniels et al. 1999). Elimination of bacteria from oyster tissues (depuration) is at least partly achieved through phagocytic action of amoebocytes (Rodrick & Ulrich 1984). The ability of oysters to successfully eliminate specific microorganisms partly depends on the species; *Vibrio vulnificus*, for example, is much more persistent in oysters than fecal pollutants such as *Escherichia coli* and *Salmo-*

nella (Perkins et al. 1980, Richards 1988, Jones et al. 1991, Tamplin & Capers 1992). Such persistence may depend on whether the bacteria have light or heavy mucopolysaccharide coats that mask the bacteria from amoebocyte recognition (Harris-Young et al. 1993, 1995, Genthner et al. 1999, Volety et al. 2001). The significantly higher *in vitro* capacity of amoebocytes of oysters from copper- and zinc-contaminated sites to kill *V. parahaemolyticus* (Fisher et al. 2003, Oliver et al. 2003) supports the seemingly ironic speculation that elevated accumulations of copper and zinc in oyster amoebocytes will reduce human health risks.

Studies of chemical influences on oyster defense processes have often demonstrated variable results. Perplexing is the fact that eastern oysters are known to thrive at some highly-polluted sites (Abbe & Sanders 1986). A few studies have linked chemical exposure of eastern oysters with higher prevalence or intensity of infectious disease (Winstead & Couch 1988, Chu & Hale 1994, Anderson et al. 1996, 1998, Fisher et al. 1999), but have never linked vulnerability to any particular defense mechanism (Oliver & Fisher 1999). Some studies have examined effects of individual chemicals on defense mechanisms of amoebocytes (Cheng & Sullivan 1984, Cheng 1988a, Cheng 1988b, Larson et al. 1989, Fisher et al. 1990, Alvarez et al. 1991, Alvarez & Friedl 1992, Anderson et al. 1992, 1994, 1997, 1998, Sami et al. 1992, 1993, Baier-Anderson & Anderson 1997) and found both suppression and stimulation of defense responses (Baier-Anderson & Anderson 2000). Several studies have exposed amoebocytes to chemicals *in vitro*, providing direct contact that may never occur if, under natural conditions, the chemicals are first bound to mucus. Studies of potential immunomodulation are complicated by the fact that chemical mixtures vary from location-to-location. Because each different chemical can elicit a different type and degree of response, it has been almost impossible to anticipate which response would predominate. The field surveys described in Section IV demonstrated enhanced activity at sites with high copper and zinc concentrations, apparently overwhelming effects of any other chemicals. The results provide a novel focus, tissue concentrations of copper and zinc, for comparing defense activities in eastern oysters.

The close association of copper and zinc accumulations with hemocyte number leads to a speculation that their availability in the ambient environment may lead to proliferation of amoebocytes. Whereas highly conjectural, the significance of this would be extraordinary. Metal stimulation of amoebocyte proliferation might parallel vertebrate immune responses that rely on antigen recognition and binding for lymphocyte proliferation. There is a possibility that increased exposure to only one metal might differentially stimulate recruitment of different amoebocyte types (AGA for copper and BGA for zinc), just as antibody-specific cells are generated by vertebrate systems. Vertebrate studies have demonstrated numerous adverse effects of copper deficiency, including reduced bactericidal activity of neutrophils, decreased macrophage activity, reduced proliferation and activity of B and T lymphocytes, and impaired cell-mediated immunity (see Kramer & Johnson 1992 for review). In fact, deficiencies in copper, zinc, and iron are all recognized to alter immunocompetence in humans and other vertebrates (Sherman 1992).

Currently, we know very little of amoebocyte proliferation in bivalves (Narain 1973). Cuénot (1891) suggested that amoebocytes might originate from special lymph glands at the base of the gills, and subsequent investigators have suggested alternate sites. Wagge (1955) and others believed that new cells were mitotically generated from existing amoebocytes. Stauber (1950) disagreed because

"of the many phagocytes examined, only one has been observed in mitosis" (p. 233). Ruddell (1971) also found only a few granular amoebocytes undergoing mitosis in histologic sections. However, Feng et al. (1977), in their studies of *C. gigas*, encountered binucleated granular amoebocytes and amoebocytes with centrioles "fairly frequently" (p. 61). They felt that Ruddell's (1971) observations might have been peculiar to activities of wound repair. If true that metals are responsible for stimulating mitotic activity, then different amounts available to oysters might well account for discrepancies in the number of mitotic amoebocytes observed in different studies.

There is an alternate possibility that "dormant" amoebocytes are activated by the presence of ambient copper. Prytherch (1934) found copper activated the pigment spot cells in mature eastern oyster larvae. Quiescent, or at least immobile until addition of copper, the cells were found to disaggregate and migrate into the hemolymph to become the first circulating amoebocytes. This "catalytic" action of copper (Prytherch 1934, p. 83) concurrently initiated larval metamorphosis. *In vitro* studies have also shown that reconstitution of cation-depleted media with seawater will initiate disaggregation and migration of adult oyster amoebocytes (Fisher & Newell 1986). Mobilization of amoebocytes has been further implied by the consistent finding of higher numbers of highly-locomotory amoebocytes from oysters collected at metal-polluted sites (Section IV). Greater locomotory activity may account for higher numbers of amoebocytes observed in hemolymph circulation. A mechanism for amoebocyte activation by copper has not been investigated, but catalytic action of copper on oxidation of glutathione has been suggested to increase cell respiration (Prytherch 1934).

The distribution of eastern oysters throughout the Gulf of Mexico and along the Atlantic coast is determined by their ability to survive and reproduce in different environmental conditions. Several have proposed that the distribution of eastern oysters is dependent on freshwater inflow because of the low and variable salinities that hinder parasite growth and high-salinity predators. Low-salinity protection from both parasites and predators has been applied in the development of a "Habitat Suitability Index" for oyster (Cake 1983, Soniat & Brody 1988). However, there is ample evidence that oysters are also successful in high-salinity environments (Beaven 1955, Lunz 1955, J.R. Nelson 1955), so an alternative reason may exist. One rationale is the availability of elements, particularly copper and zinc, from land-based sources. Terrestrial elements are carried by freshwater runoff into bays and estuaries (Reidel et al. 1995, 1998), greater volumes of water carrying them further into the receiving waters. If this dependence is found credible, then the coastal distribution of eastern oysters may be driven by a need for terrestrial elements that play critical roles in oyster physiology, including defense and nutrition (this review), shell deposition (Fisher 2004) and larval setting and metamorphosis (Prytherch 1934).

The investigations reviewed here varied widely in scope, purpose, and content. Consequently, several caveats are required. First, portions of the information reviewed may not have been intended for the purposes to which they have been applied. Even so, the pivotal studies seem well within the perceived intentions of the investigators. Although information was considered from research on several oyster species (particularly *C. virginica*, *C. gigas*, and *O. edulis*), the proposed function in defense is intended only for *C. virginica*. Adequate evidence to compare metal accumulation, adult avoidance of toxicity, and retention of accumulated metals among different oyster species was lacking. Similarly, the

review has emphasized, and is limited to, the fates and effects of copper and zinc. This focus stems from the high concentrations found in *C. virginica* and the nearly exclusive retention of these metals within the amebocytes. Other reviewers might have included tin (Orton 1923), manganese or iron (Galtsoff 1953, 1964) for consideration. These elements may serve physiologic roles similar to copper and zinc, but this is neither proposed nor defended here. Finally, because issues were wide-ranging, review of material for any specific topic has not been comprehensive. Many studies, such as those showing sublethal effects of copper or zinc exposure were not included (e.g., Cheng 1988a, 1988b, Larson et al. 1989, Anderson et al. 1994, Roesijadi 1994, Ringwood et al. 1998, Butler and Roesijadi 2001). Nor were studies depicting environmental effects on bioavailability of metals (e.g., Zamuda & Sunda 1982, Wright & Zamuda 1987, Knezovich 1994), even though this topic is clearly relevant to the subject. Despite the caveats and selective focus, the evidence presented here supports a compelling image of copper and zinc as elements highly valued, if not fundamental, for oyster defense and nutrition. Such a perspective, in spite of the obvious need for testing and validation,

provokes a need to re-evaluate many biologic, environmental, and resource issues that may be affected.

ACKNOWLEDGMENTS

Florida field survey data were obtained through the combined efforts of my colleagues, Leah M. Oliver and James T. Winstead, to whom I am grateful for many collaborations over the last decade. Thanks are also due our collaborators Edward R. Long, Aswani K. Volety, Fred J. Genthner, and Becky L. Hemmer. I am indebted to Dr. Robert S. Brown, who graciously provided both insight and material from his unpublished dissertation at Johns Hopkins University. Library material was assembled with the assistance of Sonya Doten and Liz Pinnell, and numerous graphics rendered by Stephen Embry. I am indebted to P. Chapman, W. Davis, J. Fournie, L. DiMichael, D. Shepard and C. Walker for meaningful discussions. Valuable comments and recommendations from reviewers of early versions, including L. Oliver, S. Jordan, R. Anderson, G. Roesijadi, and M. Carriker are greatly appreciated. This was prepared in support of the Gulf Ecology Division mission in Altered Habitat, and is GED contribution #1196.

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RELATIONSHIP OF AMEBOCYTES AND TERRESTRIAL ELEMENTS TO ADULT SHELL DEPOSITION IN EASTERN OYSTERS

WILLIAM S. FISHER

United States Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, 1 Sabine Island Drive, Gulf Breeze, FL 32561

ABSTRACT Freshwater runoff contains terrestrial elements from geologic deposits that may be vital to survival of eastern oysters *Crassostrea virginica*, a factor that could account for their distribution near terrestrial watersheds. Some of these elements are accumulated in granules of oyster amebocytes, which are highly mobile, diapedetic cells with widely-recognized roles in oyster defense and nutrition. Amebocytes are known to discharge antimicrobial substances from membrane-lined cytoplasmic granules into extracellular spaces, a process referred to as degranulation. A similar, if not identical, process has been described for discharge of copper and zinc from cytoplasmic granules to precipitate hemolymph proteins and initiate clotting. This review examines evidence that supports amebocyte involvement in eastern oyster shell deposition and a potential role for accumulated terrestrial elements in crystallization. It is proposed that metal-bearing amebocytes discharge inorganic elements that precipitate proteins and initiate or facilitate shell crystallization. Support for this conjecture derives partly from high concentrations of terrestrial elements in adult shell, changes in shell morphology at metamorphosis, hypothesized mechanisms for shell repair and pearl formation, and the absence of other known mechanisms for shell crystallization.

KEY WORDS: oysters, *Crassostrea virginica*, marine bivalves, copper, metals, contaminants, amebocytes, leucocytes, hemocytes, larval setting, larval metamorphosis, shell deposition, metal toxicity, oyster management, oyster reef restoration

INTRODUCTION

Population distributions of eastern oysters *Crassostrea virginica* near terrestrial watersheds has led to a general impression that low or variable salinity is vital to their survival (Cake 1983, Soniat & Brody 1988, Berrigan et al. 1991). However, freshwater runoff contains numerous mineral elements from geologic deposits that could perform significant functions in oyster physiology. Copper and zinc, for example, are accumulated to extraordinarily high concentrations in eastern oysters, even in the absence of anthropogenic sources. There is no known physiologic function for such high concentrations of these terrestrial metals, but it has been proposed that they are actively assimilated and stored in amebocyte granules for antimicrobial activities related to defense and nutrition (Fisher 2004). Both metals are accumulated in the amebocytes and calcareous shell of oysters, a unique distribution that indicates a link between amebocytes and deposited shell, the initial and final repositories for the metals.

Because amebocytes (blood cells, hemocytes) are known to recognize, locate, ingest, and digest a variety of biotic and abiotic foreign particles (phagocytosis), the role of amebocytes in microbial defense has been widely investigated (Metchnikoff 1891, Yonge 1926, 1937, 1946, Stauber 1961, Cheng 1975, 1977, 1996, Feng et al. 1977, Feng 1988). A hypothesis that copper and zinc are instrumental in amebocyte antimicrobial activities was generated from evidence that the metals are avidly concentrated by eastern oysters from low ambient concentrations, and are retained in amebocyte granules for relatively long periods of time despite available elimination mechanisms (Fisher 2004). There is evidence that copper and zinc, stored at high concentrations in amebocyte granules, can be discharged into phagosomes for intracellular killing or into extracellular spaces (degranulation) at wound sites for microbicidal action and clot formation (Ruddell 1971, Sparks 1972, Brown 1975, Ruddell & Rains 1975). Here, discharge of

metals is proposed as a mechanism to initiate or facilitate shell crystallization.

The importance of the oyster shell is indisputable; shell structure, strength, and growth are critical factors in oyster survival. According to Melbourne R. Carriker, an authority on eastern oyster shells and shell formation,

"Because the oyster lacks an internal skeleton, its valves serve as an exoskeleton, providing support for the soft internal organs and preventing collapse of the mantle cavity. Consequently, vital physiologic functions of the mantle cavity, i.e., circulation of seawater, gaseous exchange, discharge of gametes, and removal of pseudofeces and fecal and catabolic wastes, can be carried out without impediment. Valves also protect internal organs from mechanical impacts, osmotic stress, and many predators, and from desiccation in the intertidal zone." (Carriker 1996, p. 102).

The greatest attributes of shell, its strength, and stability, are obtained through crystallization, which binds disassociated elements into an inflexible crystalline lattice as the shell is deposited. In this overview, evidence is presented to link shell deposition with activities of amebocytes and with the discharge of metals from amebocyte granules that may serve to initiate or facilitate crystallization. As crystallization proceeds, the metals are irreversibly bound and accrue to high concentrations in the shell. Amebocyte involvement in shell deposition is supported by the existence of high concentrations of terrestrial metals in deposited shell, differences in larval and adult shell structure, and the potential role of metal deposition in shell crystallization. Like defense and nutrition, deposition of shell serves an important survival function for oysters, meriting the expenditure of energy necessary to accumulate and deposit the metals. A dependence of oysters on terrestrial metals could account for their exclusively estuarine and near-coastal distribution.

AMEBOCYTE INVOLVEMENT IN SHELL DEPOSITION

The chemist M. E. Fremy found that molluscan shell was composed primarily of carbonate of lime which, when treated with hydrochloric acid, left a

"...brilliant, felt-like residue, not soluble in hot water, like horn or gelatin, and insoluble in alcohol and ether."
(Woodward 1866)

Fremy named the organic substance *conchioline* and, because it retained its structural integrity after acid treatment, demonstrated that the shell was not a single network, but two distinct components. Fremy's discovery led to the hypothesis that an organic framework of conchioline (or conchiolin, Fig. 1) was formed from cells or layers of membrane (Bowerbank 1844, Carpenter 1844, Woodward 1866), and lime was then deposited upon this framework. The concept of a two-step process has endured (Crenshaw 1980, 1982, Wilbur & Saleuddin 1983), as summarized by Carriker (1996),

"Shell formation occurs in two major steps: (a) ion transport, protein synthesis, and secretion by mantle epithelial cells, and (b) physicochemical processes in the extrapallial space in which mineral crystals are nucleated, oriented, and grow in intimate associate with the secreted organic matrix." (Carriker 1996, p. 123)

Mantle tissue has long been considered the organ responsible for both steps of shell deposition.

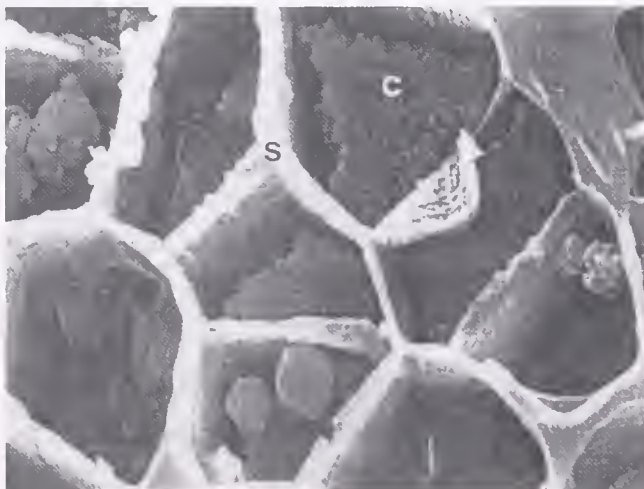


Figure 1. Conchiolin matrix of *Crassostrea virginica*. Acid treatment or standing sea water removes the mineral core (c) of shells to reveal the sheaths (s) of organic matrix that are insoluble and acid-resistant. Figures from Carriker (1996, Figs. 99 and 100), courtesy of Maryland Sea Grant and with permission of the author.

"The growth of shell results from the deposition of crystalline material within an organic matrix of conchiolin by a thin sheet of tissue called the mantle, which lines the inner surface of the shell." (Tsujii et al. 1958, p. 276)

"Biocrystals develop in the extrapallial space within the soluble matrix (between the inner shell surface and the outer mantle epithelium) from mineral and organic material passed through or originating in the mantle epithelium." (Carriker 1996, p. 122)

This widely held perception is drawn from the close association of mantle tissue with new shell surfaces and the highly secretory nature of mantle epithelium. Yet, amebocytes, often high in number, are also present in the extrapallial spaces of bivalves (Wagge 1955, Beedham 1965, Bayne 1983) including eastern oysters (Paillard et al. 1996). And, whereas the mantle is considered the primary tissue for shell deposition, amebocyte participation has often been implicated. Based primarily on the suggestions of Wagge (1955), several reviews of bivalve amebocytes have concluded that amebocytes participate in shell repair (e.g., Narain 1973, Cheng 1981, 1984, Fisher 1986). Despite this connection, there has been, until recently, no direct evidence of amebocyte involvement in shell repair or shell deposition in eastern oysters.

Early Observations

There is fortuitous evidence that bivalve amebocytes participate in shell deposition. Although Woodward (1866) conformed to the hypothesis that mantle tissues were responsible for shell, he inadvertently described something that might be interpreted differently:

"If we examine any immature shell, we shall find the lip, or growing margin of the shell, much thinner than the rest of the shell, and, indeed, quite soft. The mucus of this soft, growing edge is found to contain granules, which are, in fact, incipient cells destined to unite and form the calcareous matter of the future shell-wall." (Woodward 1866, p. 250)

It is not clear from this description how 'incipient cells' might fuse into shell material, but there can be little doubt that Woodward observed granular cells in the mucus of the growing shell edge that he believed were responsible for shell calcification. He anticipated that these cells were derived from mantle epithelium, but his characterization could also describe migrating amebocytes.

In another example, Bevelander and Martin (1949) described the *in vitro* culture of excised mantle tissue from *Pinctada radiata*. They reported that strips of mantle could secrete mucus, epithelial cells, blood cells, and fibrous material (conchiolin) which formed shell-like material.

"Cultures maintained for longer periods, 4-6 days, frequently exhibit the presence of crystal aggregates typical of those found in normal or regenerating shell. In the majority of these preparations the crystal formation occurs some distance away from the mantle edge, and usually in an exudate consisting of mucus." (Bevelander & Martin 1949, p. 614)

This short abstract (Bevelander & Martin 1949) is significant because it demonstrates, at least for *P. radiata*, that both conchiolin formation and crystallization can occur without direct contact with mantle epithelial cells. Instead, the abstract noted blood cells (amebocytes) in the mucus where new shell was being formed.

Shell Repair In Land Snails

One of the most compelling arguments for amebocyte participation in shell formation stems from the insights of Wagge (1951, 1955), who studied shell repair and deposition in the land snail *Helix aspersa*. Wagge (1955) found that during new shell deposition, calcium carbonate was passed from mantle epithelial cells into the extrapallial cavity to form a colloidal gel that crystallized into shell. But he questioned how mantle cells could perform repairs throughout the entire shell.

"To what extent do the mantle epithelial cells act in situ when they are laying down new layers of shell, or absorbing calcium from other parts of the shell? They could not lie in positions in which they were found in certain regenerates and at the same time have remained an integral part of the mantle epithelium." (Wagge 1955, p. 45)

His principal concern was that stationary mantle epithelial cells could not reach remote or restricted sites. He also questioned how these cells could absorb material (e.g., calcium) from one site and transport it to a different site for shell deposition. Finally, he wondered how secretions that emanated grossly from mantle tissue could avoid forming new shell throughout the mantle cavity, even if repairs were needed at only a single site. All of these issues were resolved by his observation of mantle epithelial cells that were capable of ameboid activity.

"The foregoing account has shown that mantle epithelial and connective tissue cells are able to: (1) absorb calcium, (2) move it to a damage area, (3) secrete the organic and crystalline framework of the new piece of shell. With freely moving cells the physico-chemical reaction can take place directly within the restricted area under repair, and not generally throughout the whole of the extrapallial fluid." (Wagge 1955, p. 48)

The presence of ameboid cells alleviated Wagge's concerns over proposed mechanisms of shell repair, and led him to wonder whether similar mechanisms were also used in formation of new shell.

"This fact, i.e. that certain mantle cells are capable of independent movement, helps considerably in the understanding of the part played by the mantle during shell formation, although whether there is as much independent movement of cells from the mantle during the formation of new shell, as there is during repair, is not known." (Wagge 1955, p. 44)

Many of Wagge's conclusions were formed by his earlier experiments (Wagge 1951) that showed migrating epithelial cells were responsible for secretion of both the organic membranes and the crystals of calcium carbonate within the calcospherites (conchiolin compartments). Although these observations were made on *Helix*, they formed the basis of later assertions that amebocytes are involved in molluscan shell repair (e.g., Narain 1973, Cheng 1981, 1984, Fisher 1986). They also provide a rationale and a model for amebocyte involvement in new shell deposition.

Pearl Formation and Deposition of Nacre

Wagge was not the first to recognize amebocyte participation in shell deposition. Early bivalve studies established that pearls were composed of material identical to, and formed in the same manner, as the nacreous inner lining of their shells. Louie Boutan (1923) attributed to Raphael Dubois (1901) the first true investigation into the process of pearl formation. The essence of Dubois' work on

Mytilus edulis was recalled in Boutan's extensive overview of natural and cultured pearls:

"La nacre, dit-il, n'est pas, comme on l'a répété jusqu'à ce jour, le produit d'une sécrétion glandulaire. Son mécanisme de formation est plus complexe. L'épithélium externe du manteau des Mollusques nacrifères sécrète le squelette organique de conchyoline. Les interstices de ce dernier sont comblés par des éléments migrants calcarifères, qui traversent l'épithélium sécréteur par un phénomène de diapédèse. La substance inorganique (carbonate de chaux) des éléments migrants est nécessairement mêlée à une certaine quantité de matière organique ayant constitué la substance vivante ou bioprotéine du plastron calcarifère." (Boutan 1923, p. 23)

Boutan (1923) emphasized that the importance of Dubois' work was his description of a two-step process in pearl formation: an organic layer deposited by the mantle epithelium and the limestone calcification provided by migratory cells. Pearls, according to Dubois, were formed by *"un charpentier et un maçon"*. Boutan (1923) was not certain whether the migrating cells originated as ectoderm (from the basement membrane of mantle epithelial cells) or were mesodermal cells that migrated across the mantle epithelium (Fig. 2). Regardless, he seemed quite certain that ameboid cells played an essential role in the crystallization of both shell nacre and pearls, the former stimulated by normal growth processes and the latter by the abnormal presence of a foreign object.

"En résumé, la structure de la perle et de la nacre paraît dépendre uniquement du concours de deux éléments dis-

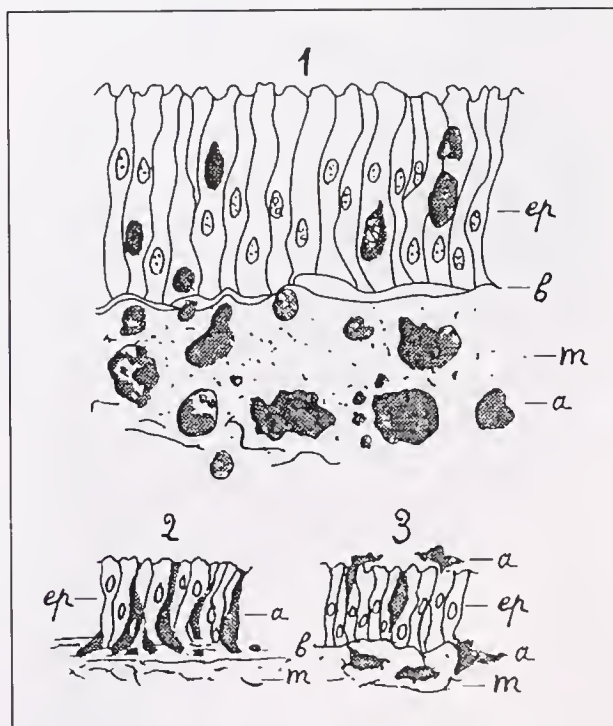


Figure 2. Drawings to show (1) a section of the epithelium forming a pearl sac, including the epithelial cells *ep*, basement membrane *b*, mesoderm *m*, and amebocytes *a*; (2) the basement membrane of the epithelium as a potential site of origin for amebocytes; and (3) the mesoderm as a potential site of origin for amebocytes, and their migration to the surface of the epithelial layer to calcify the pearl. Drawings are by A. Rubbel, reproduced from Boutan (1923, Fig. 1).

tuets, fournis, l'un par l'épithélium, l'autre par un élément cellulaire, dont l'origine reste incertaine. On comprend facilement que cette structure puisse être influencée par l'état de l'épithélium normal dans le cas de la sécrétion de la nacre, anormal dans la cas de la formation de la perle fine, aux dépens d'un sac clos dérivé de la épithélium." (Boutan 1923, pp. 24-25)

He showed that the cells forming the pearl sac were extensions of the mantle epithelium (see Fig. 2) that appeared to be in a state of chronic inflammation (Boutan 1923, p. 22). The findings of Dubois and Boutan were no doubt instrumental in Wagge's (1955) conclusion that migrating ameboid cells were essential contributors to shell formation.

Recent evidence has emerged that directly links amebocytes to deposition of shell (Mount et al. 2004). Scanning electron microscopy and x-ray microanalysis were used to document the formation in certain granular amebocytes (refractory cells) of calcium carbonate crystals that were then deposited at the site of shell repair. There is no evidence yet of how the crystals are formed or whether all granulocytes have the capacity to form them. Crystals were observed in amebocyte cytoplasm and later distributed at the shell mineralization front. This may be the first direct evidence for eastern oyster amebocyte involvement in shell deposition.

ACCUMULATIONS OF METAL IN EASTERN OYSTER SHELL

Eastern oyster shells contain high concentrations of terrestrial metals. There have been many reports of high metal content in eastern oyster soft tissues (see Fisher 2004), but shells also accumulate high amounts. For example,

"Soft parts of oysters showed on the average about six times the zinc concentration of the shells (wet weight basis). But since the soft parts accounted for only $19.5 \pm 4.1\%$ of the total live weight of the oysters, however, the shells contained nearly 45% of the total zinc in this group of oysters." (Wolfe 1970, p. 52)

Although values reported for oysters vary (Table 1), metal concentrations are consistently found to be higher in shells than in the surrounding seawater. Ferrell et al. (1973) estimated that many metals (Pb, Hg, Cd, Zn, Cu, and Cr) in *C. virginica* shells were more than a thousand times higher than seawater concentrations.

TABLE 1.

Concentrations of metals in eastern oyster shell reported by different sources.

Metal	Concentration ($\mu\text{g g}^{-1}$)	Source
Aluminum	200	Smith & Wright 1962
Cadmium	8–10	Ferrel et al. 1973
Chromium	2–4	Ferrel et al. 1973
Copper	3	Smith & Weight 1962
	43–70	Ferrel et al. 1973
Iron	180	Smith & Wright 1962
Lead	36–41	Ferrel et al. 1973
Manganese	110	Smith & Wright 1962
Mercury	0.09	Ferrel et al. 1973
Zinc	2	Smith & Wright 1962
	25	Wolfe 1970
	2–8	Windom & Smith 1972
	5–9	Ferrel et al. 1973

"The trace metals are present in substantially higher concentrations than in sea water, thus illustrating the degree of accumulation possible during the secretion of the shell by the mantle of the oyster." (Ferrell et al. 1973, p. 315)

This assessment has been upheld by others (Carriker et al. 1980, 1982, 1991, Simkiss 1983).

Metals Acquired During Shell Deposition

There is an apparent consensus, evident from the earlier citation, that incorporation of metals in oyster shell occurs as the shell is deposited. The consensus, at least for zinc, seems to be correct. Wolfe (1970) found little difference in zinc concentrations of living oyster shell with that of geological deposits of oyster shell.

"Zinc may be released from the shell matrix very slowly, so that shells represent a significantly large reservoir of zinc with a slow turnover in the environment." (Wolfe 1970, p. 52–53).

Slow release from shell implies that zinc is tightly bound in the shell matrix and is not in rapid flux with ambient concentrations. This means that zinc is incorporated during shell deposition, when it can be tightly bound in the crystalline matrix.

There is, though, some flux of zinc between existing shell and the environment. Studies have shown a rapid uptake and exchange between ^{65}Zn in shell and in the water column (Chipman et al. 1958, Fitzgerald & Skauen 1963, Wolfe 1970, Romeril 1971), seemingly due to adsorption and release driven by ambient concentrations. Chipman et al. (1958) noted that addition of ^{65}Zn adsorbed to shells initially, but if oysters were moved to untreated water the isotope disassociated from the shells to establish a new steady state with the surrounding water. Although these studies demonstrate the ability of zinc to adsorb to shell, adsorbed amounts are probably minor in relation to those amounts bound in the shell matrix.

There are two general mechanisms that could bind metals during shell deposition: (1) metals could be incorporated directly from the ambient environment over time, or (2) metals could be incorporated as needed from internal stores. The first possibility presumes that oyster shells grow sufficiently slow to concentrate metals available in the ambient milieu. Even though ambient concentrations are usually low and often variable, many elements form carbonates that could be directly incorporated (Ferrell et al. 1973). Certainly, this is possible if, as some have argued, shell accumulation of trace metals is.

"... partly a coincidental result of poor discrimination by the biologic mechanism for calcium uptake and shell deposition." (Wolfe 1970, p. 55)

But a similar argument for high concentrations of copper and zinc in oyster soft tissues was found lacking. Rather than inadvertent uptake, accumulation of copper and zinc is more likely a dedicated physiologic process to acquire the metals for nutritional and defense activities of amebocytes (Fisher 2004).

Metals Provided by Amebocytes

The second possibility for metal incorporation in shell presumes that some mechanism is available to concentrate metals, store them safely (i.e., without toxic effect), and discharge them into the organic matrix as shell is deposited. Amebocyte storage of metals fulfills each of these conditions. Copper and zinc are sequestered in membrane-lined granules of amebocytes to prevent

toxicity, then discharged into extracellular spaces as needed for antimicrobial activity (Ruddell 1971). Although existing data are largely limited to copper and zinc, it is very likely that other metals found in shell, such as iron, manganese, and tin, are also stored by amebocytes (Orton 1923, Galtsoff 1938, 1953, 1964). If so, then amebocytes may be an internal storehouse for a variety of metals ultimately incorporated into shell. This second alternative is reinforced if the metals are incorporated to serve some function in shell deposition, architecture or integrity. As such, their availability would become a limiting factor in shell growth and necessitate a stable and readily available source of metals, especially at times of rapid growth. This alternative would present a distinct advantage over accrual from the low and variable amounts provided by ambient waters.

Higher Metal Concentrations in Adult Shells

It has been demonstrated that adult shells of eastern oysters have higher concentrations of metals than larval shells. Carriker et al. (1991), using proton-induced x-ray emission data showed that metal content in calcite (adult) shell, particularly prismatic forms, was higher than in aragonite (larval) shell. Although the studies were performed entirely on adult oysters, the section of shell containing the myostracal scar retains the aragonitic shell structure of larvae. These data confirmed earlier results (atomic absorption spectrophotometry, Carriker et al. 1980) that compared prismatic calcite shells with the foliated structure of the internal shell surface. Although the contents varied, the prismatic calcite shells contained more zinc, iron, and other metals than the myostracal and foliated shell types (Table 2). These studies link higher metal content to calcite shell morphology, the form of shell that is produced by juveniles and adults after larval metamorphosis.

METAMORPHIC CHANGES IN AMEBOCYTES AND SHELL STRUCTURE

The foregoing sections have described observations of amebocyte involvement in shell deposition and the high accumulation of metals bound in the shell matrix. Because at least some of these metals are avidly assimilated and concentrated in amebocytes, which have mechanisms for safe storage and discharge, it is hypothesized that amebocytes deposit the metals into newly forming shell. Adult eastern oyster shells have higher concentrations of metal than larval shells so, if there is an association among amebocytes, metals, and shell structure, then there must be a funda-

mental difference in how shell is deposited before and after metamorphosis.

Structural Differences Between Larval and Adult Shells

A relatively unique difference exists between adult and larval shells of eastern oysters; the larval (prodissoconch) shell is aragonite (highly regular arrangement) and the adult shell is calcite (irregular, prismatic arrangement). Transition from one shell form to the other occurs simultaneously with larval setting and metamorphosis. Stafford (1913) described the visible distinction between the prodissoconch shell, which remains the same size once the oyster has set, and the juvenile/adult shell that grows from the margin of the larval shell (Fig. 3). The structural demarcation of the two shell types is termed the *metamorphic line* (Carriker 1996).

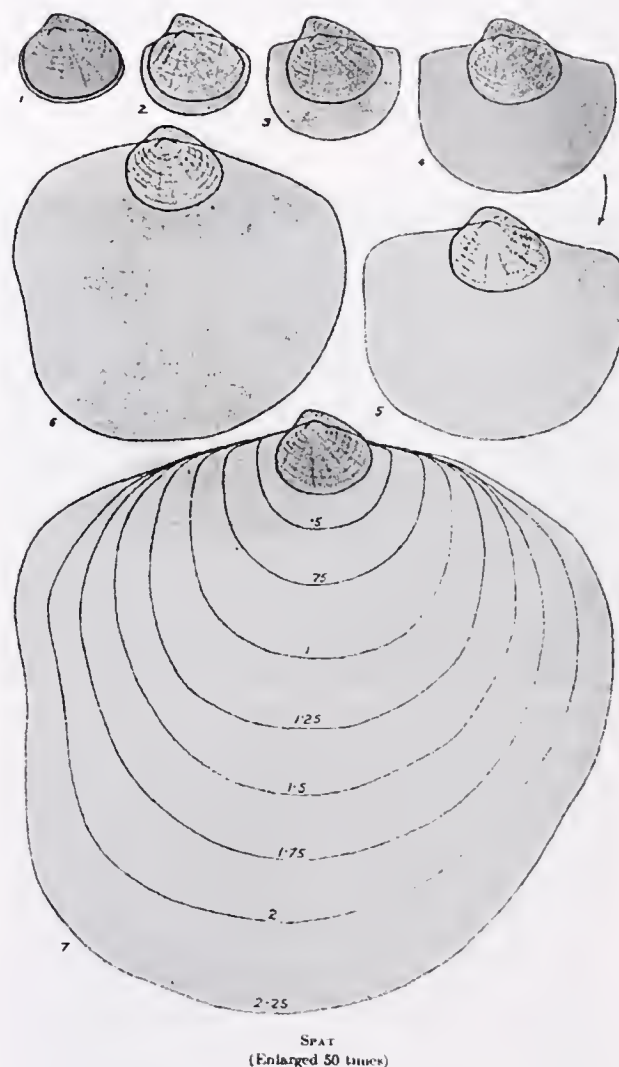


Figure 3. Growth of the shell of *Crassostrea virginica* from the time of settlement through early spat growth. The metamorphic line delineates a transition from the aragonite shell of larvae, shown in (1) with a thin rim of newly deposited spat shell, to the calcite shell of juveniles and adults. Sizes of spat in the drawings range from 0.379 mm (1) to 2.25 mm (7) in length. The largest spat drawn (7) is eight larval shells superimposed so that the shells coincide to represent a spat at different stages in its growth from 0.5–2.25 mm length. Reproduced from Stafford (1913; Plate II).

TABLE 2.

Mean distribution of elements in different eastern oyster adult shell types, analyzed by proton-induced X-ray emission and reported as % by elemental weight in ppt (from Carriker et al. 1991, Table 3). These selected, but representative results demonstrate greater metal content in prismatic shell than foliated and myostracal scar shell. Myostracal scar shell is considered representative of larval aragonite shell structure. Carriker et al. (1991) report similar data on several valve types and shell sections, and examine 15 elements, including Na, Mg, S, Cl, Ti, Cr, Br and Sr not shown here.

Shell Type	Al	Si	Ca	Mn	Fe	Cu	Zn
Prismatic	11.09	21.21	908	0.543	4.782	0.024	0.122
Foliated	<0.50	<0.50	961	0.153	0.601	<0.010	0.088
Myostracal	1.55	<0.50	975	0.012	0.042	<0.010	0.012

Beyond the metamorphic line, shell structure is prismatic calcite on external portions (Fig. 4) and foliated calcite on internal portions. The reason for the structural change at metamorphosis is not understood, although Stenzel (1964) believed it to be because,

"... calcite is more stable and much less subject to leaching in the sea water and because calcite can be secreted more economically than aragonite. Secreted calcite fills a larger volume per mole than aragonite. Adult oysters need a thick shell for defense against predators." (Stenzel 1964, p. 156)

Wise (1970) suggested that substitution of calcite shells in place of aragonite shells represented an evolutionary change driven by expediency. Apparently, animals secreting calcitostracum can simultaneously form multiple laminae in the organic matrix, thereby speeding the process of shell crystallization.

Regardless of evolutionary purpose, this change at metamorphosis must accompany a fundamental modification in the manner that shell is deposited. Carriker (1996) marveled at the probable physiologic alterations this would entail.

"Change in shell microstructure and mineralogy during metamorphosis probably could not occur without some

modification of the secretion of the shell materials by the mantle epithelium. Epithelial cells, which in planktonic larval stages secreted aragonitic granules, quite suddenly are reconstituted in the early spat to form a microstructure and orientation of strikingly different biocrystals at the rim of the valves." (Carriker 1996, p. 97)

Yet, evidence is lacking that indicates any modification of the mantle epithelium:

"Of lively interest is the subtle cellular differentiation that must occur in mantle margins at metamorphosis. Are microstructural and microchemical changes evident at the cellular and molecular level by modern scientific technology? Unfortunately, knowledge of these metamorphic changes is essentially nonexistent." (Carriker 1996, p. 157)

A reason for this lack of knowledge may be that the transformation of shell structure has little to do with cellular differentiation in the mantle. Instead, the transformation may be driven by changes in the activities of amebocytes.

Relation of Copper to Larval Metamorphosis

A review of functional roles for terrestrial metals in eastern oysters could not be complete without considering the research of Herbert F. Prytherch, Director of the US Fisheries Biological Station in Beaufort, North Carolina. During the late 1920s, Prytherch performed studies at Milford, Connecticut, to describe the process and events of oyster setting and metamorphosis. Like many others, Prytherch (1929, 1934) noted that eastern oysters were invariably distributed along inshore areas and near the mouths of rivers, locations that exhibited a continuous mixing of seawater and river water. Heaviest oyster setting occurred during neap tides when river flow and lower salinities dominated, and could extend seaward during periods of high river discharge. His laboratory experiments demonstrated that salinity had little influence on setting success, although it did affect the time required (Fig. 5). More important to successful setting, he found, were the components of river water.

"When the salinity was reduced with river water instead of distilled water, the larvae gave a positive setting reaction which indicated that some change or substance had been introduced by the river water that served to stimulate and induce setting." (Prytherch 1934, p. 68)

He later found that substance to be copper, which induced setting "almost immediately" (15–30 sec) when a 0.5 cm square piece was dipped into sea water.

Prytherch (1934) attempted to find other conditions and chemicals that might induce larval setting. He found no effect of temperature, salinity, pH, oxygen content, carbon dioxide content, water pressure, or current velocity. Neither neutral salts (chlorides, sulphates, carbonates, and nitrates of sodium, potassium, magnesium, and calcium) nor inorganic elements (Ag, Al, Ba, Fe, Mn, Ni, Pb, Sn, and Zn), other than copper, had any effect (Table 3).

"The only element in the heavy metal series that produced a positive setting reaction was copper in the form of a pure metal or as a dissolved salt. This heavy metal was found to be effective and initiated almost immediately the setting process in concentrations as low as 1 part copper to 25 million or 50 million parts of sea water. In the controls the larvae lived for a week or two but eventually died after failing to set or metamorphose. However, if copper stimulation was applied during this period the larvae would set and develop into healthy spat." (Prytherch 1934, p. 77)

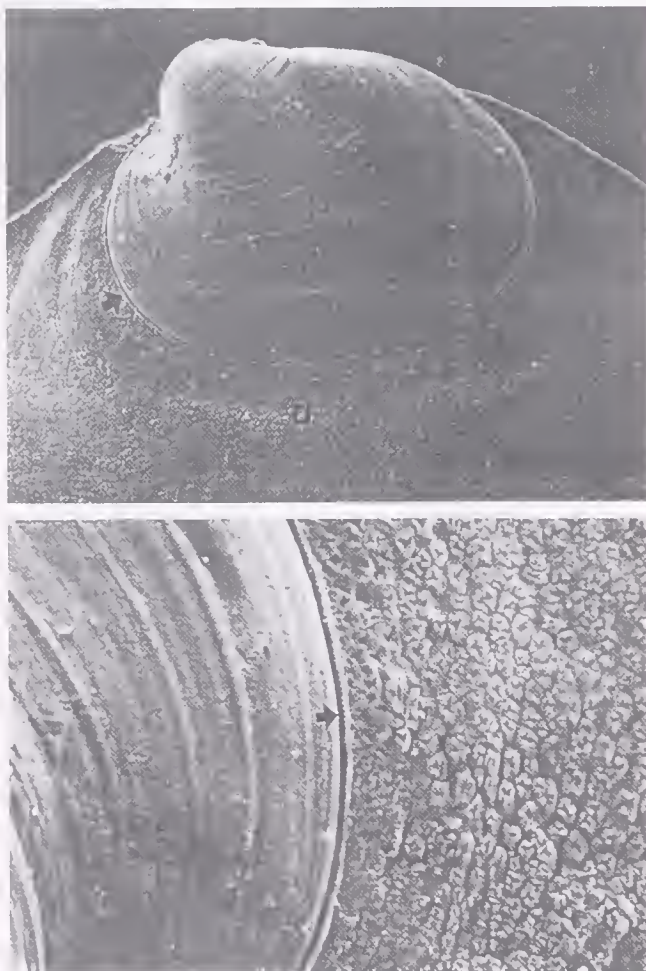


Figure 4. Top: Settled spat of *Crassostrea virginica* showing the metamorphic line (arrow) that distinguishes shell generated during the larval (prodissoconch, p) stages from that generated after oyster setting (dissoconch, d). Bottom: Metamorphic boundary (arrow) showing differences in shell structure. From Carriker (1996, Figs. 40 and 41), courtesy of Maryland Sea Grant and with permission of the author.

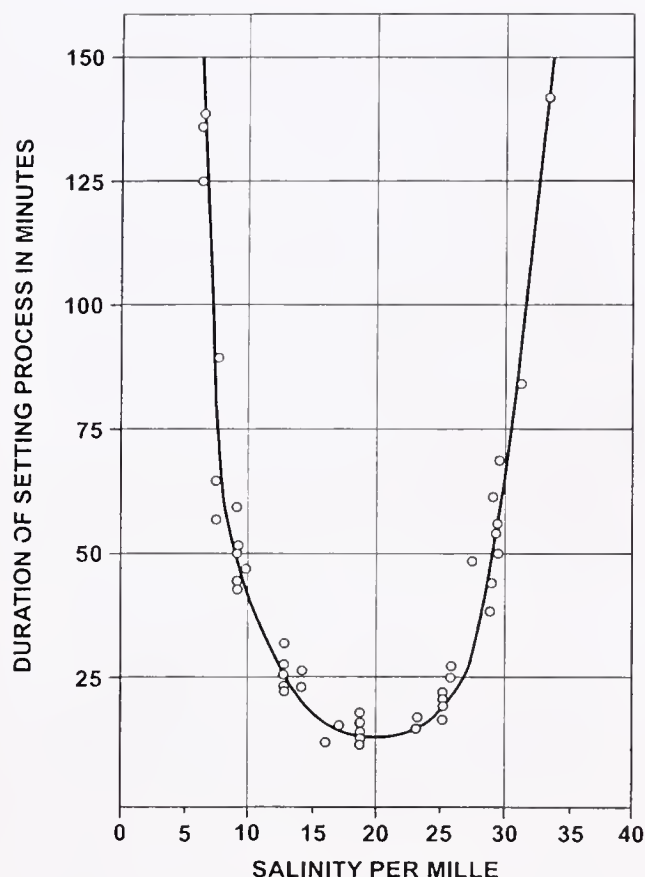


Figure 5. Effect of salinity on duration of eastern oyster setting (Prytherch 1934). Late-stage oyster larvae were acclimated 1-3 days at salinities ranging from about 4-40 ppt prepared from stock sea water (28 ppt salinity) by evaporation or dilution with distilled water. Larvae were induced to settle with addition of very small amounts of copper, and the time required to set was monitored. No setting occurred after 4 h for larvae maintained in salinities below 5.6 ppt or above 32.2 ppt. Data are representative of over 450 larvae tested in this manner. Redrawn from Prytherch (1934, Fig. 9).

He later refined his estimate of the required copper concentration to range from 50 to 600 $\mu\text{g L}^{-1}$, and demonstrated greater setting success at the high end of the range.

Relation of Copper and Pigment Spots to Metamorphosis

Once an eastern oyster larva has set, numerous morphologic changes occur, including disappearance of the pigment spots, atrophy of the velum and foot, rapid growth of the gills and mantle, development of the adductor muscle, and an increase in heart rate. Among these transformations is the appearance of circulating amebocytes in the hemolymph. The amebocytes originate, according to Prytherch (1934), from the pigment spots, blue-green aggregates of cells (Fig. 6) that disappear at metamorphosis.

"When setting is in progress, the pigmented cells in each spot gradually enlarge, separate, and one or two break away and are carried forward by the blood stream into the mantle. After [larval] attachment, the pigment spot breaks apart more rapidly; and all the cells become distributed in a short time into the circulatory system, as the process of transformation of the larva is in full swing. Each pigment spot consists of approximately 300 cells which, after sepa-

TABLE 3.

Metals tested by Prytherch (1934) for ability to induce setting in eastern oyster larvae. Each concentration of each element was tested at 15, 20, and 25 ppt salinity at both pH 7.5 and pH 8.0.

Element	Form	Concentration Range ($\mu\text{g L}^{-1}$)	Larval Setting
Aluminum	$\text{Al}_2(\text{SO}_4)_3$	1-20	Negative
	AlCl_3	1-20	Negative
	Metallic	1 min-6 hrs ^a	Negative
Barium	BaCO_3	0.05-5	Negative
	BaSO_4	0.05-5	Negative
	BaCl_2	0.05-5	Negative
Copper	$\text{CuCO}_3 \cdot \text{Cu}(\text{OH})_2$	0.05-1	Positive ^b
	CuSO_4	0.05-1	Positive
	CuCl_2	0.05-1	Positive
	Cu_2CO_3	0.05-1	Positive
	CuOH	0.05-1	Positive
	Cu_2Cl_2	10 sec-5 min ^a	Positive
	$\text{Fe}_3(\text{SO}_4)_3$	0.1-10	Negative
Iron	FeCl_3	0.05-20	Negative
	FeCO_3	0.1-10	Negative
	FeSO_4	0.1-10	Negative
	FeCl_2	0.1-10	Negative
	Metallic	1 min-24 hrs ^a	Negative
	PbCO_3	0.1-10	Negative
	PbSO_4	0.1-10	Negative
Lead	PbCl_2	0.1-10	Negative
	Metallic	1 hr-24 hrs ^a	Negative
Manganese	MnCO_3	1-50	Negative
	MnSO_4	1-50	Negative
	MnCl_2	1-50	Negative
Nickel	NiCO_3	0.05-5	Negative
	NiSO_4	0.05-5	Negative
	NiCl_2	0.05-5	Negative
Silver	Metallic	1 min-1 hr ^a	Negative
	AgCl	0.5-1	Negative
	AgNO_3	0.5-1	Negative
Tin	Metallic	1-24 hr ^a	Negative
	SnCl_4	0.5-5	Negative
	SnCl_2	0.5-5	Negative
	SnSO_4	0.5-5	Negative
Zinc	Metallic	1-24 hrs ^a	Negative
	ZnCO_3	0.5-5	Negative
	ZnSO_4	0.5-5	Negative
	ZnCl_2	0.5-5	Negative
	Metallic	1 min-6 hrs ^a	Negative

^a Metallic forms were immersed in the sea water for the durations noted.

^b Copper concentrations above 500 $\mu\text{g L}^{-1}$ were toxic to larvae; lower concentrations of all copper salts stimulated setting.

ration, resemble in many respects the leukocytes of the spat and adult oyster. The breaking up of the pigment spots is an important and necessary preliminary step to metamorphosis, and requires copper as the initial stimulus." (Prytherch 1934, p. 86)

Prytherch demonstrated that pigment spot dispersal and metamorphosis ceased if copper were removed from the medium, even for newly set organisms.

The pigment spots have often been called "eyespot" and, because they appear only near the end of larval maturation, their presence has been used to signal mature larvae that are ready for setting ("eyed" larvae). Although the source has not been investigated, the increasing visibility of pigment spots with larval devel-

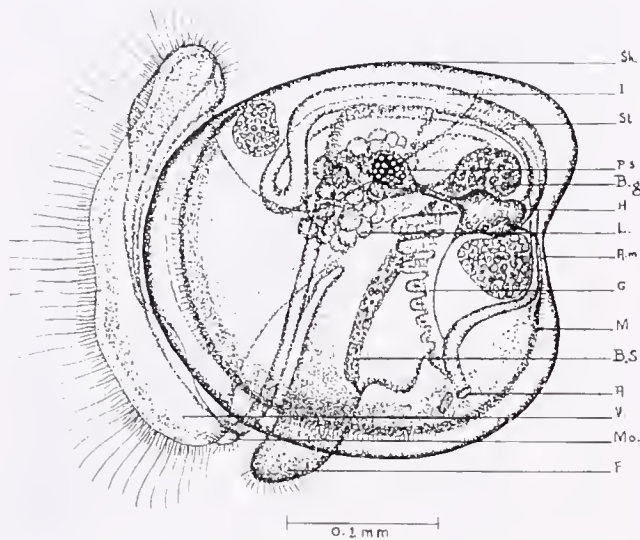


Figure 6. Left side of a *C. virginica* larva showing the pigment spot (P.S.) connected by a vessel to the heart (H.). At metamorphosis, Prytherch (1934) has observed the approximately 300 cells that comprise each pigment spot to disperse into the blood vessels and become the circulating amebocytes of spat. The pigment spots have also been termed 'eye spots', although evidence of sensory capacity is lacking. Other organs include shell (Sh.), intestine (I.), stomach (St.), byssal gland (Bg.), liver (L.), posterior adductor muscle (A.m.), gills (G.), mantle (M.), byssal secretion (B.S.), anus (A.), velum (V.), mouth (Mo.) and foot (F.) Drawing reproduced from Prytherch (1934, Fig. 4) with permission of *Ecological Monographs*.

opment is probably due to increasing concentrations of accumulated copper. As copper is accumulated, the amebocytes gradually become more visible—just as they become visible on mantle surfaces, hearts, and blood vessels when carrying high concentrations of copper (see Fisher 2004). Prytherch (1934) recognized this likely association:

"Metamorphosis follows the release into the circulatory system of the numerous cells, containing a blue-green pigment, which compose each pigment spot. According to Galtsoff and Whipple (1930) there is a definite relation between the green pigment in adult oysters and the amount of copper present. These authors found that the intensity in green color was in proportion to the copper content of the oyster and that histologically the copper is located in the green leukocytes." (Prytherch 1934, p. 87)

For Prytherch, the significance of copper as an integral part of metamorphosis was based on his belief that copper was essential for respiration. He postulated that copper porphyrin served as a respiratory catalyst similar to iron porphyrin in vertebrates. This link with respiration provided Prytherch a rationale for the unique copper requirement he had discovered, and led him to view the release of the pigment spot cells into the blood stream as a key event in the life of the oyster.

"Though many morphologic changes occur in the transformation of the larva into an adult oyster, those which appear to be of greatest biologic significance are concerned with the breaking up of the two pigment spots after ingestion of minute amounts of copper." (Prytherch 1934, p. 87)

Although respiratory pigments have not been found in eastern oysters, the association of copper with setting and metamorphosis

is highly intriguing. Yet, it has received little attention in subsequent literature.

The link between pigment spot amebocytes and metamorphosis was disputed by Cole (1938) who believed *O. edulis* pigment spots were sensory eye spots. This he apparently concluded from their bilateral symmetry and histologic characteristics, because there were no descriptions of neural connections (Stafford 1913, Prytherch 1934, Cole 1938). He believed that larvae developed sensitivity to light to ensure setting in dark, protected locations, a hypothesis that is still unresolved (see Kennedy 1996). Instead of dispersal, Cole (1938) believed that the "eyespot" cells disappeared at metamorphosis because they were phagocytosed by amebocytes. Cole's position was adopted by Galtsoff (1964) for *C. virginica* and, except for one passing note (Vinogradov 1953), the possibility that pigment spots are immobile amebocytes has apparently been abandoned.

Nonetheless, there is indirect evidence that copper influences the activation and activity of oyster amebocytes. Field studies have shown that oysters with high tissue concentrations of copper had higher numbers of locomotory amebocytes and higher rates of amebocyte locomotion (Fisher et al. 2000, Oliver et al. 2001). Also, aggregation and dispersal of oyster amebocytes can be controlled *in vitro* by the presence or absence of cations in the medium (Fisher & Newell 1986). Without cations in the seawater, amebocytes drawn from oyster hemolymph remained immobile and aggregated. Dispersion did not occur until cations were reintroduced. This observation is similar to that of Prytherch (1934), who found mobility and dispersal of pigment spot amebocytes was dependent on the presence of copper.

TERRESTRIAL ELEMENTS AND SHELL CRYSTALLIZATION

Bevelander & Nakahara (1969) substantiated an early hypothesis that the organic matrix (conchiolin) of marine bivalves was elaborated across the growth surface of new shell in advance of mineralization. The matrix formed compartments that contained substances necessary for crystallization. In pelecypods, crystal initiation is believed to occur randomly at "nucleation sites" within the matrix compartments (Wise 1970). Once initiated, crystals grow and coalesce to form the larger crystals and shell patterns observed microscopically (Tsujii et al. 1958, Wise 1970, Carriker 1996).

"It is thought that formation of an organic matrix probably precedes formation of biocrystals. The matrix in all likelihood is involved in the nucleation, orientation, type, and size of biocrystals, and in providing the surface to which soluble matrix attaches and functions as a nucleating surface. It has been suggested that once crystal nuclei are formed, biocrystals grow inorganically." (Carriker 1996, p. 122)

Evident throughout the literature, however, is a lack of detail related to mechanisms of nucleation and crystal initiation. Galtsoff (1964) related hypotheses of nucleation by carbon dioxide (De Waele 1929) and citrate (Steinhardt 1946), but was not convinced that either was true. Later, Towe and Hamilton (1968) suggested that the organic matrix became polymerized into layered sheets that provided nucleation sites for epitactic precipitation of calcium carbonate. This is probably a reasonable reflection of the proposal of Bevelander and Nakahara (1969), who suggested that the organic matrix provided a "proper environment" for spontaneous crystallization.

Yet, it became clear that if spontaneous precipitation led to

crystal growth, then there must be some means to halt the process and confine mineralization within the conchiolin compartments. Microstructure analysis has indicated that crystallization occurs independently within each compartment (Wise 1970). A possible resolution was suggested by Tsujii et al. (1958):

"It is apparent that some mechanism exists for maintaining the height of the matrix and the crystal surface at approximately the same level. Movements of the mantle probably accomplish this by distributing secreted matrix. If the crystal faces become higher than the matrix boundaries through crystal growth, the mantle movements would fill in the depressions between crystals with matrix. Excess material would be moved across the crystal faces." (Tsujii et al. 1958, p. 277)

This explanation, however, seemed to imply a second secretion of matrix from the mantle, somehow guided by muscular movements of the mantle to specific sites exhibiting excess crystal growth. Carriker (1996), apparently recognizing the inherent conflict of a matrix material that both initiated and limited crystal growth, attempted to refine the concept.

"As a stratum (lamina) of biocrystals attains a certain thickness, apparently a layer of insoluble matrix is added and then sclerotized in situ, stopping further thickening of the layer. As a consequence, the inner surface of valve thickens incrementally. The mechanism for this stratification, involving mantle secretory cells and the complex of secretions in the extrapallial space, is unknown." (Carriker 1996, p. 122)

As implied, Carriker found no evidence for deposition of a second layer of matrix or its sclerotization.

The issue described here is not confined to eastern oysters. In most shell-forming organisms it is generally believed that the organic matrix is somehow responsible for regulation of crystallization (Crenshaw 1980, Mann 1983, Weiner et al. 1983), whether through initiation or limitation of crystal growth, or influence over crystal type and shape (Wheeler et al. 1988). Many apparently believed that the matrix was involved in crystal initiation (Wheeler & Sikes 1984), so it was unexpected when Wheeler et al. (1981) found organic matrix, resolubilized from shells of *C. virginica*, actually inhibited *in vitro* crystallization. This evidence led Wheeler et al. (1987) to question the applicability of *in vitro* indicators and to suggest an alternative to the existing "calcium binding" concept for crystal nucleation. They proposed instead "crystal binding", in which soluble matrix actually binds to and stabilizes clusters of ions "which form and dissolve repeatedly in metastable physiologic solutions" (p. 958-959). Despite these efforts, it seems that the role of matrix in calcification remains an enigma.

Overlooked in these investigations was the fact that amebocytes carrying high concentrations of accumulated copper, zinc, and probably other cationic metals traverse the surface of the conchiolin matrix. This fact affords an alternative mechanism: Amebocytes on the matrix surface could discharge stored metals onto the matrix to precipitate proteins (or even salts) and initiate crystallization. A mechanism for such a process was previously proposed by Brown (1975) for initiation of clotting in eastern oyster hemolymph. Brown suggested that metals discharged from amebocytes (degranulation) precipitated proteins in the hemolymph plasma to create a finely granular gel, or clot, capable of trapping bacteria. Metal-laden amebocytes in mucus of the mantle cavity may similarly mobilize to sites of shell deposition (whether for

repair or new growth) and discharge stored metals that precipitate proteins for the formation of crystals.

Such a mechanism would explain two phenomena, crystallization and the transfer of metals from amebocytes to shell. Recent research, however, has demonstrated that crystals are formed within granular amebocytes and then released onto the surface of the matrix (Mount et al. 2004). It is conceivable that metal-protein precipitation is responsible for the formation of crystals within the amebocyte. Alternatively, metals could be dispersed by degranulation onto the conchiolin surface after the release of crystals to facilitate crystal growth or alter the crystal structure for strength or efficiency (i.e. formation of calcite rather than aragonite). Certainly, these new findings indicate that nucleation may not be so simple as metal-protein precipitation on the conchiolin surface. Nonetheless, it is very probable that granular amebocytes at the site of shell growth are simultaneously involved in both shell deposition and the transfer of metals to shell.

Proposed Role of Amebocytes and Terrestrial Elements in Shell Crystallization

Based on the earlier information, a role for amebocytes and terrestrial elements in adult shell crystallization is proposed. During larval development, amebocytes accumulate copper (and possibly other elements) and store them in membrane-lined cytoplasmic granules. The gradual accrual of copper eventually endows amebocytes with a blue-green pigmentation that makes them visible (pigment spots). Once sufficient copper is accumulated, amebocytes are mobilized into hemolymph circulation and dispersed throughout the tissues. This coincides with, and may actually trigger, metamorphosis. Mobile amebocytes can then migrate to the mucus of the mantle cavity and participate in shell deposition. Metal stored in amebocyte granules may be involved in crystal formation within the amebocyte or may be discharged onto the matrix of newly forming shell to precipitate proteins. In either case, metals are irreversibly bound in the shell as it forms and concomitantly change the shell structure from an aragonite (larval) to calcite (adult) shell.

BIOLOGIC RELEVANCE

On the assumption that high ambient and accumulated concentrations of terrestrial metals must be harmful, a perception has evolved that metals are sequestered in oysters solely to detoxify them until they are eliminated. This is not true however, for copper and zinc in eastern oysters. Much of the relevant information amassed during the last century supports a hypothesis that copper and zinc are actively captured, sequestered, and retained by oyster amebocytes for specific physiologic purposes. These purposes may include larval setting and metamorphosis (Prytherch 1934), nutrition, and defense (Fisher 2004). Zinc, copper, and other terrestrial metals may also be essential for shell construction, as described here. If so, several aspects of oyster biology are affected.

Amebocytes seem to have an extraordinary capacity to perform in a wide range of physiologic functions, including nutrition, defense, excretion, metamorphosis, and shell repair and deposition. This is because of their unique ability to traverse epithelial barriers and move throughout the soft tissues and shell matrix. This was emphasized by Wagge (1955):

"Growth, regeneration, and repair are other functions greatly assisted by amebocytes and certain problems in

invertebrates are more easily resolvable if one envisages the possibility that this primitive amoebocytic transport system can move with speed to a certain area, act efficiently, and increase its numbers both from the reactivation of resting cells and by further budding or division of the appropriate tissues." (Wagge 1955, p. 74–75)

Because of the high visibility of nutrition and defense, amoebocytes have sometimes been perceived to exist only in the alimentary tract and hemolymph. Yet, amoebocytes are active throughout the oyster, including the mucus and shell.

Importance of Terrestrial Elements

Amoebocytes have another unique characteristic, the ability to capture and store terrestrial elements at high concentrations without toxic effect. These elements may be needed at relatively high concentrations to perform various physiologic functions, including shell deposition. High concentrations of copper and zinc may be needed to provide flexibility for competing needs in defense and nutrition (Fisher 2004). High concentrations of these and other metals may also be needed for shell deposition. A balance must exist between shell growth and tissue growth, the latter being neither consistent nor predictable because it is dependent on widely fluctuating environmental factors (e.g., temperature, salinity, or food availability). If elements essential to shell growth are not available at the time of tissue growth, then opportunity is lost. If, however, high accumulations of precipitating metals are retained in reserves (amoebocytes), then the oyster could deposit new shell at every available opportunity.

The composition of shell indicates that various elements are incorporated during shell deposition, including aluminum, copper, iron, manganese, zinc (Smith and Wright 1962), cadmium, chromium, lead, and mercury (Ferrell et al. 1973). Some elements may be incorporated directly from the water column, but those at high concentrations most likely come from an internal storehouse such as the cytoplasmic granules of amoebocytes. It is not clear how many elements are stored in amoebocytes. Orton (1923) reported that copper, zinc, and tin were accumulated in the amoebocytes of *O. edulis*, whereas Galtsoff (1938, 1953, 1964) reported iron and manganese in amoebocytes *C. virginica*. The content of amoebocyte granules, despite differences in assimilation and retention, must ultimately be determined by the composition in the local environment, albeit at lower concentrations (Galtsoff 1964, Ferrell et al. 1973, Carriker et al. 1982, 1991, Simkiss 1983).

It is unknown which elements might serve to precipitate proteins in shell matrix or even serve as crystallizing nuclei. Some effects of ambient concentrations of metals have been investigated. Shuster & Pringle (1969) found excellent shell growth for oysters exposed to elevated concentrations of copper, but poor shell growth of those exposed to cadmium. Some reports have noted that oyster shells formed in polluted environments were thin, relatively flaky, or multilayered compared with oysters from less contaminated sites (Frazier 1976, Okazaki & Panietz 1981). Frazier (1976) believed that shell thinning was linked to effects of copper and cadmium on shell calcification enzymes. Although these data are few, conflicting, and largely qualitative, they could indicate that metals exert an influence on shell deposition that ultimately affects shell quality. If competent shell structure (i.e., strength and stability) depends on metal composition, then deviations in metal composition may adversely affect shell quality.

This possibility is relevant to effects of a high-profile environmental toxicant, tributyltin (TBT), which once was considered a

successful antifouling agent in marine paints. Thickened and areolar shells of *C. gigas* (Fig. 7) in the Gironde River, France, were the first warning that TBT carried a high environmental impact (Alzieu et al. 1980, 1982, Waldo & Thain 1983). TBT can impose sex changes ("imposex") and reproductive sterility in gastropods from chronic exposures as low as 2 ng L⁻¹ (Gibbs & Bryan 1994). Alzieu (1986) believed that the effect of TBT on *C. gigas* shell was on mechanisms of calcification.

"Perturbations of the calcification mechanisms of oysters cultivated in the Atlantic Ocean have been wide-spread since 1979. The abnormal shells were thickened and their cut showed numerous chambers fill[ed] in with a jelly. . . . The components of [this] jelly do not [bind] with Ca²⁺ or with HCO₃⁻, and when added to [a] solution of CaCO₃ slow down or prevent the formation of crystals of CaCO₃." (Alzieu 1986, pp. 1131–1132)

It may be that the jelly component observed by Alzieu was actually the conchiolin matrix deposited for new shell that could not crystallize because the normal complement of metals had been supplanted by TBT. In water exposures of eastern oysters to TBT (Fisher et al. 1999), relatively high concentrations were localized

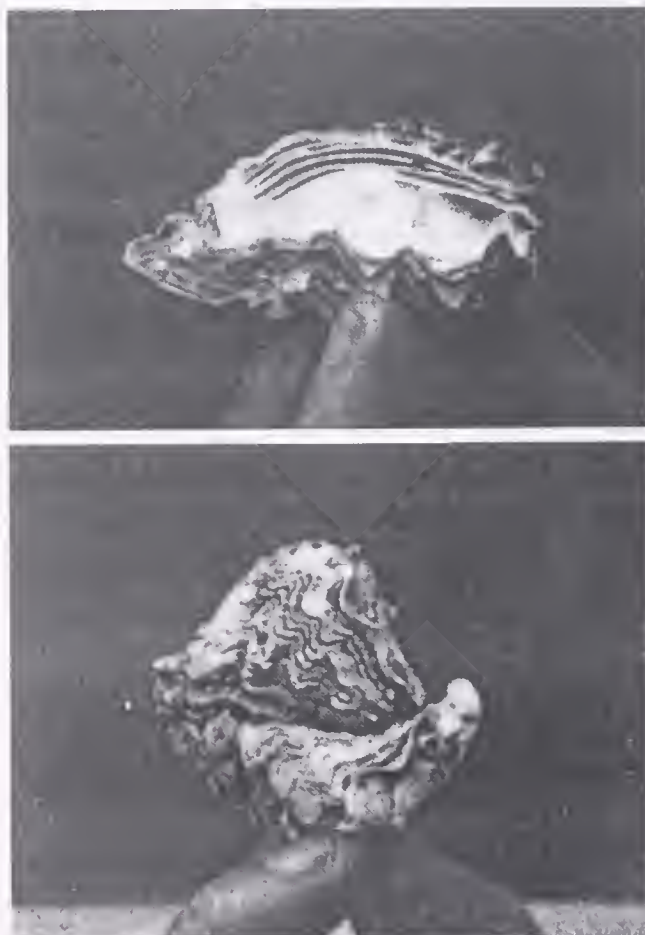


Figure 7. *Crassostrea gigas* shells are deformed by exposure to tributyltin, an anti-fouling paint used in marine paints. *Top:* Shell cut open to show chambering in the upper valve. *Bottom:* Chambering sufficiently severe to create a 'ball-shaped' oyster. These malformations occur in *C. gigas*, and apparently do not occur in *C. virginica* or *Ostrea edulis*. Photographs reprinted from Alzieu (1986, Figs. 2 and 3) with permission of the author.

in amebocytes (unpublished data). Abnormal shell development found for *C. gigas* does not seem to extend to *O. edulis* (Waldock et al. 1983, Alzieu 1986) or *C. virginica* (Rice et al. 1987). It is interesting, albeit perplexing, that environmental regulations to reduce TBT in estuaries may have created a greater demand for copper-based marine antifouling paints (Claisse & Alzieu 1993). As noted before, copper may not be toxic to adult eastern oysters, but is toxic to larvae and can inhibit setting and metamorphosis at high concentrations (Prytherch 1934, Fisher 2004).

Copper Stimulation of Metamorphosis

According to Prytherch (1934), many of the physiologic changes that occur at metamorphosis are a consequence of activation by copper, which he believed oxidized glutathione and elevated cellular respiration. He postulated (p. 85) that copper in the water column was ingested into the stomachs of oyster larvae, dissociated by low pH, and then distributed, apparently via hemolymph, to various organs to stimulate metabolic activity. It is equally possible, however, that copper from the water column is gradually accumulated in the cytoplasmic granules of amebocytes. Once a threshold is surpassed, either in the amount or concentration of copper, the amebocytes are activated and disperse into hemolymph and tissues, where they may effect activation of other cells by release of copper. Prytherch (1934) described only the amebocytes that comprised the pigment spots, but amebocytes (coelomocytes) are also known to exist in the visceral cavity of larvae (Elston 1980a, Elston 1980b). These, too, may be accumulating copper during larval development and could participate in metabolic stimulation of cells. The ability of amebocytes to discharge copper to extracellular spaces (degranulation) provides a capacity to distribute the element to different cells and organs.

Metamorphosis is abrupt, rather than gradual, so it is more likely that a stimulating substance (e.g., copper), is released from highly concentrated internal stores, rather than extracted from low or variable ambient concentrations. If copper is a trigger for metamorphosis, then the amebocyte threshold for copper accumulation could represent the amount needed to ensure activation of not just amebocytes, but of all organs undergoing metamorphic change. Amebocytes, once distributed throughout oyster tissues, could distribute copper to multiple organs simultaneously. It is worth noting that copper in spawned eggs of *C. virginica* were found to contain very similar concentrations of copper (Greig et al. 1975), even

though concentrations from the parents could vary (Table 4). This implies that copper concentrations in embryos may be tightly regulated.

Influence of Freshwater Inflow on Oyster Distribution

Distribution of eastern oysters throughout the Gulf of Mexico and along the Atlantic coast is often associated with reduced salinities (Cake 1983, Soniat & Brody 1988). Reduced salinity is believed to relieve pressure from recent disease epizootics caused by *Perkinsus marinus* (Andrews & Ray 1988) and *Haplosporidium nelsoni* (Ford 1985), and from predation by drills, sponges, starfish, and other high-salinity predators that have coexisted with oysters for millennia (Loosanoff 1955, Lunz 1955, Hopkins 1956, Menzel et al. 1966). Counter to this model is the fact that eastern oysters are not always restricted to regions with reduced salinity. Beaven (1955) identified two setting patterns in the Chesapeake Bay, patterns that appear fairly consistent throughout the range of eastern oysters.

"One type occurs where rich runoff enters directly into high salinity waters producing a body of water exhibiting a fairly steep salinity gradient. Oyster beds in such areas are subject for brief periods to salinities low enough to prevent the establishment of many of the predators and fouling agents that hinder spat survival in waters of a more sustained high salinity.... A second type of high setting area consists of a semi enclosed body of water where the salinity is fairly uniform and where there is a comparatively slow rate of exchange of water overlying the oyster beds." (Beaven 1955, p. 34)

The second type of setting area resembles those described for the tidal creeks of South Carolina (Lunz 1955) and oceanic populations in Virginia and Delaware (Nelson 1955). Reduced salinity, then, is not necessarily required for oyster setting or the ultimate survival of eastern oysters.

An alternate explanation for distribution patterns of eastern oysters was implied by Prytherch (1934), who described a dependence on recurring influxes of copper from the watershed. Successful setting required that larvae were exposed to appropriate concentrations of copper at least once at maturity, if not intermittently throughout their development. Any discrepancies or deviations from this, whether stemming from tides, currents, drought, freshwater diversion, sedimentation, lack of resuspension, or the mere absence of appreciable copper in geologic deposits, would seemingly eliminate successful setting. Watershed runoff with sufficient volume (i.e., streams and rivers) can generate flows that allow oyster setting far and deep into the receiving water. But in areas of low-volume runoff, oyster setting may be restricted to sites directly adjacent to land. At these (usually intertidal) sites, copper concentrations may be maintained by proximity to its source (land) and possibly from resuspension of particulate matter.

A dependence on copper might help to explain the sometimes confusing year-to-year variability in larval settlement that has been recorded at the same locations over several years (Collins 1889, Prytherch 1929, Hopkins 1935, Andrews 1955, Beaven 1955, Nelson 1955). Successful setting would require that mature larvae, spawned at different locations and times throughout the spring and summer, must co-occur in time and space with clean substrate (cultch) and sufficient concentrations of copper in a seawater milieu. Most oyster setting areas are probably subject to day-to-day, even hour-to-hour, variability in the availability of copper.

Unlike adult oysters, larvae are susceptible to high water con-

TABLE 4.

Concentrations ($\mu\text{g g}^{-1}$ dry weight) of selected elements in adults and spawned eggs from *C. virginica* collected at two sites in Connecticut, Housatonic River (HR) and Branford (BR), as reported by Grieg et al. (1975).

Element	Site	Adults	Eggs
Cadmium	HR	28.1	<1.6
	BR	15.6	<1.2
Copper	HR	2,208	28.9
	BR	1,260	27.8
Lead	HR	<4.2	<10
	BR	7.1	<9.9
Silver	HR	12.1	<1.6
	BR	16.4	<1.2
Zinc	HR	10,460	82.4
	BR	8,300	65.9

centrations of copper (Prytherch 1934, Calabrese et al. 1977). Hence, the amount of copper available to stimulate setting and metamorphosis is critical. Prytherch (1934) found the greatest setting intensity occurred at copper concentrations that were nearly toxic. Freshwater inflows contaminated by anthropogenic activity might elevate copper, or other chemical pollutants, to concentrations that are toxic to embryos or larvae. Adult oysters may not be vulnerable because copper and other cations are probably bound in the mucus of the mantle cavity, protecting susceptible tissues (Fisher 2004). Binding in mucus would also protect amoebocytes that, *in vitro*, are sensitive to high copper concentrations (Butler & Roesijadi 2001).

Survival of spat, juvenile and adult oysters may require terrestrial elements other than copper. In fact, a copper requirement may have evolved as a surrogate to ensure that oysters settle in areas with an ample supply of terrestrial elements. Zinc is the most highly concentrated element in eastern oyster amoebocytes and may be important in phagocytosis for defense and nutrition (Fisher 2004) as well as in shell deposition. Accumulations of other elements, though not as dramatic as copper and zinc, are also quite high and may serve additional roles in oyster physiology.

Effects of Altered Freshwater Inflow

Copper, zinc, and other metals that may be critical to setting, growth, or survival of eastern oysters are provided by terrestrial runoff and river inflow to estuaries and coastal zones. How much and when these elements arrive at oyster beds may determine recruitment, health, or toxicity to oysters. Consequently, human activities that alter the availability and timing of water runoff and inflow will likely alter the health and sustainability of eastern oyster populations. Agriculture and urbanization are major activities that affect the input terrestrial metals into coastal zones and estuaries (Browder & Moore 1981). Some agricultural practices, such as deforestation, will increase the amount of sediment (and presumably elements) into the water. Other agricultural practices combine with urbanization to deplete coastal zones of fresh water. Dams and canals, whether for agriculture, drinking water, sewage disposal, or generation of hydroelectric power, alter the volume and timing of freshwater flows to estuaries.

Sammy M. Ray, widely recognized for his research with *Perkinsus marinus* disease, has often proposed retaining natural freshwater flows to reduce disease incidence in oyster-growing regions.

"Oysters will flourish only in areas where sufficient freshwater inflows occur on a regular basis." (Andrews & Ray 1988)

Whereas emphasis has been placed on avoidance of predators and disease, eastern oyster success and distribution may also be a reflection of the evolutionary advantage gained by their ability to accumulate and use high concentrations of otherwise toxic metals. Any alteration of metal availability, which occurs with alteration of the quantity or timing of freshwater flows, denies the oyster this competitive advantage.

The eastern oyster has for over a century supported a major commercial fishery. The sustainability of the resource has been severely threatened by heavy fishing pressure from the turn of the 20th century and by disease in its latter half. We have slowly realized that oysters and their habitats (reefs) provide valuable ecosystem features, including biodiversity (Wells 1961, Breitburg et al. 1995, Breitburg 1999, Posey et al. 1999), water filtration (Haven & Morales-Alamo 1970, Officer et al. 1982, Newell 1988, Harsh & Luckenbach 1999), biodeposition (Haven & Morales-Alamo 1966, Dame et al. 1984), and nutrient mineralization (Pomeroy & Haskin 1954, Dame et al. 1989, Dame & Libes 1993, Dame 1999). Because of the reliance of fish on oyster reefs for refuge and prey items, some consider oyster reefs to be essential fish habitat (Coen et al. 1999). Most states or regions with an economic or ecologic interest in oysters have already begun attempts to conserve and restore productive reefs (Luckenbach et al. 1999). The probable dependence of oyster setting, growth, and survival on copper and other terrestrial elements will influence decisions on which reefs to protect and which sites to restore.

ACKNOWLEDGMENTS

Thanks are due to Melbourne Carriker, Guri Roesijadi, Leah Oliver, Steve Jordan, and Robert Anderson for useful comments on early versions of this report. Robert S. Brown provided material from his unpublished dissertation at Johns Hopkins University. Library material was assembled by Sonya Doten and Liz Pinnell, graphic representations prepared by Stephen Embry, and translations graciously provided by Tere McMahon. Sea Grant of Maryland provided copies of Figures 1 and 4. Thanks are extended to R. Elston, C. Walker, D. Shepard, J. Fournie, L. DiMichael, W. Davis and P. Chapman for valuable discussions and information. The editor of JSR kindly allowed updates to this report after its acceptance. This was prepared in support of the Gulf Ecology Division mission in Altered Habitat, and is GED contribution #1197.

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QUARANTINE OF OYSTER SHELL CULTCH REDUCES THE ABUNDANCE OF *PERKINSUS MARINUS*

DAVID BUSHEK,^{1,*} DONNIA RICHARDSON,² M. YVONNE BOBO² AND LOREN D. COEN²

¹USC-Baruch Marine Field Laboratory, Georgetown, South Carolina 29442; and ²South Carolina Department of Natural Resources, Marine Resources Research Institute, Charleston, South Carolina 29412

ABSTRACT Oyster shell is the preferred substrate for replanting oyster beds and restoring oyster reefs. If pathogens remain viable in tissues attached to shell, then planting shell may inadvertently serve as a vector for pathogen transmission. Limited local shell sources may exacerbate the problem by increasing the risk of spreading novel strains into new areas if shell is derived from other regions. In South Carolina, the primary source of oyster shell is currently the Gulf of Mexico, where the protozoan oyster pathogen *Perkinsus marinus* (Mackin et al. 1978) has been problematic. Although *P. marinus* is present in South Carolina waters, different strains exist in the two regions. Given the detrimental effects of *P. marinus* on oysters, protocols to minimize its spread via planting of shell are needed. We conducted a short-term, replicated experiment to follow changes in *P. marinus* abundance in oyster tissues by placing whole, intact oysters or shucked oysters in shell piles. The amount of oyster tissue present and parasite abundance declined precipitously after one month and was virtually eliminated by three months. Parasite persistence was dramatically longer in whole, unshucked oyster tissues as compared with those associated with shucked oysters. Viability of parasites after one month was, however, unclear. The results support the recommendation that the quarantine of shell for one month or more can dramatically reduce the potential risk of spreading *P. marinus* when planting oyster shell (= cultch) from other geographic areas. This recommendation is applicable to virtually any region, but several parameters such as effects of climatic conditions and shell pile configuration warrant further investigation as does the persistence of other pathogens on shell piles.

KEY WORDS: Dermo, disease transfer, oyster, *Perkinsus*, quarantine, restoration, SC, shell planting

INTRODUCTION

It is now widely recognized that oyster reefs are valuable habitat for a wide variety of organisms (Coen et al. 1999, Luckenbach et al. 1999) and that oyster resources can be enhanced through directed oyster reef restoration programs (Coen & Luckenbach 2000). As a result, oyster reef restoration programs have expanded rapidly throughout the United States in recent years. Unfortunately, oyster shell needed to rebuild reefs is generally scarce in those areas where restoration is needed most. Alternatives to shucked oyster shell cultch include clam shell, whelk shell, coal ash by-products, fossil shell, old porcelain material, marl (sedimentary CaCO_3), and similar materials (Luckenbach et al. 1999), but the reef-building material of choice is oyster cultch (Coen & Luckenbach 2000, and references therein). The most economical source of plentiful shell for restoration programs along the Atlantic coast of the United States is from the Gulf of Mexico. A major question related to the use of out-of-state shell is its potential to function as a vector of disease and non-native species, especially at a time when invasive species and new disease strains are being introduced around the globe (e.g., see Mann 1983, Naylor et al. 2001, Chapman et al. 2003, NAS 2003).

One potential risk of using oyster cultch from the Gulf of Mexico for replanting and restoring beds and reefs on the Atlantic Coast is the protozoan oyster pathogen *Perkinsus marinus*, which causes dermo disease in the Eastern oyster *Crassostrea virginica* (Gmelin, 1791). This parasite is abundant throughout the Gulf of Mexico (Soniati 1996) and along the Atlantic Coast (Burreson & Ragone Calvo 1996, Ford 1996, Bobo et al. 1997), but different strains inhabit or are predominant in different regions (Bushek & Allen 1996, Reece et al. 1997, Reece et al. 2001). The introduction of additional, possibly more virulent, parasites is a real threat to

any state's oyster populations. Therefore, the development and use of protocols that minimize the survival or persistence of *P. marinus* is warranted.

Little information exists concerning the survival of pathogens on cultch (shell) or the treatment of cultch prior to replanting to prevent the transmission of diseases. Preliminary work revealed that desiccated tissues (adductor muscle) found attached to oyster shell sampled from a large shell pile in Louisiana contained *P. marinus* parasites—putatively identified via body burden assay (Bushek et al. 1994) with Ray's fluid thioglycollate medium (RFTM) (Ray 1966). Subsequently, a sample of fresh shell (aged less than one month) on a shell pile from South Carolina contained whole intact oysters (i.e., not shucked) and dehydrated tissues as above on single valves. Large oysters (>50 mm) contained 12.7 parasites per gram, whereas smaller oysters, which were more commonly found whole and intact, contained 44 parasites per gram wet tissue (Bushek 1997, Bushek 1998). Though these parasite numbers are not particularly high, Mackin (1962) reported that 100–500 cells could initiate an infection, whereas Valiulis (1973) reported the initiation of infections with as few as ten cells. It is entirely possible that the number of parasites had already declined substantially in the oysters examined by Bushek (1997, 1998) while on the shell piles before collection, but the initial infection intensities of the oysters examined were unknown. Furthermore, there was no way to ascertain the viability of the parasites. If those parasites detected are not viable, then they pose no threat to populations where the shells are planted. On the other hand, viable parasites moved with shells planted to restore or enhance oyster populations could actually exacerbate oyster production problems simply by increasing the number of parasites available to infect oysters.

In light of these observations, we undertook an experimental study with the following four objectives: (1) to monitor the persistence or elimination of *Perkinsus marinus* surviving in tissues that remain attached to oyster shell in shell piles; (2) to compare *P.*

*Corresponding author. E-mail: bushek@hsrl.rotgers.edu

marinus persistence in shucked oysters versus whole (initially live, unshucked) oysters (the worst-case scenario); (3) to provide better guidance on how to treat imported shell to protect local oyster resources; and (4) to make management recommendations as appropriate.

MATERIALS AND METHODS

This study applied an experimental approach using small (approximately 100 U.S. bushel) shell piles constructed by South Carolina Department of Natural Resources (SCDNR) Marine Resources Division and seeded with *Perkinsus marinus* infected oysters from the Gulf of Mexico. Ninety *P. marinus*-infected oysters (mean shell height \pm 1 SD = 74.8 \pm 11.1 mm) were collected from Confederate Reef, West Galveston Bay, Texas, on March 19, 2002, and shipped overnight to SCDNR's Marine Resources Research Institute in Charleston, South Carolina. At the time of collection, salinity was 26 psu and surface water temperature was 25°C. Shell height and whole weight were recorded for each oyster. On March 22, 2002, 45 oysters were shucked, wet meat weight determined, and the meats divided into two equal samples. Care was taken to divide tissues equally during all dissections. One half of the soft tissues were removed for time zero sampling and the other half was returned to the valves which were then loosely held together with rubber bands to form a container for placement in experimental shell piles as described below.

Time zero samples (March 22, 2002) were further divided into three equal samples, each comprising about one-sixth of the entire oyster. One portion was processed for *P. marinus* body burden analysis using standard protocols (Bushek et al. 1994, Fisher & Oliver 1996). The second portion was processed identically, except without the RFTM incubation step for reasons outlined below. The third portion was used to determine tissue dry weight.

The non-RFTM incubation sample was used as a conservative validation of parasite viability. During RFTM incubation, viable *P. marinus* enlarge forming a prezoosporangium that possesses a NaOH-resistant cell wall (Choi et al. 1989) and stains blue-black with Lugol's iodine (Ray 1952). The total number of *P. marinus* cells in the entire oyster was then estimated from the number of prezoosporangia present in the sample processed for body burden analysis with RFTM incubation. We assumed that enlargement in RFTM indicated cell viability at the time of sampling. Nonviable cells do not enlarge or develop the NaOH-resistant cell wall and are therefore digested during the NaOH incubation step. It is possible, however, that some *P. marinus* parasites form prezoosporangia in moribund and decaying oyster tissues (Ray 1954, Mackin 1962), but this phenomenon is poorly documented, and the viability of such cells is unknown. By processing a portion of each oyster with RFTM and a portion without RFTM incubation, we attempted to determine the proportion of parasites that were responding to the RFTM and therefore viable. The viability of any parasites that enlarged prior to RFTM incubation could not be determined. Hence, our estimate of viability was conservative.

Three experimental piles (each roughly 100 U.S. bushels) were constructed from existing sources of recently shucked Gulf and South Carolina oyster shell. In March 2002, three replicate plastic mesh bags, each containing five whole, unshucked oysters and five of the shucked oysters processed above, were buried approximately 0.5-m deep in each shell pile. Oysters were assigned to treatments (shucked or whole) and to bags based on size to ensure an approximately equal size distribution for subsequent sampling. The total number of parasites added to each pile via shucked or

whole unshucked oysters was estimated from the body burden samples taken above. Because only half of the tissue from shucked oysters was added to shell piles, the total number of parasites added to a pile in shucked oyster tissues was calculated as half the sum of the total body burden estimates. We assumed parasite burdens were similarly distributed among whole unshucked oysters. Because whole unshucked oysters were randomly distributed within size classes, we assumed that each pile received an equivalent number of parasites in total from these oysters. StowAway temperature data loggers from Onset Corporation were deployed within the piles to monitor internal temperature logging every 30 min. A fourth data logger recorded air temperature at the site for a portion of the experiment.

One bag was selected and removed from each pile after 31, 73, and 115 days (Fig. 1) to quantify and process remaining tissues for *P. marinus* body burden as described above with the following differences. On day 31 (April 22, 2002), tissues remaining in shucked oysters were divided into two equal samples and processed for *P. marinus* body burden, one with RFTM incubation and one without as described above. These samples were considered to represent 25% of what would have remained had the entire oyster been placed in the pile. Whole oysters were shucked and tissues divided into three equal portions for RFTM body burden analysis, non-RFTM body burden analysis and dry weight determination. On day 73 (June 3, 2002), samples were processed identically to day 31 except dry weight was not determined; instead, one portion was used in an attempt to isolate and culture *P. marinus* *in vitro*. Parasites were purified as described by Chu and La Peyre (1993) and cultured as described by Dungan and Hamilton (1995). On day 115 (July 15, 2002), samples were processed similarly except whole oysters were divided into two samples only for RFTM and non-RFTM *P. marinus* body burden estimation. Temperature loggers were downloaded each time that oysters were sampled and then redeployed.

Data were analyzed by comparing descriptive statistics and plots of relevant parameters over time for shucked and unshucked oysters. A three-way analysis of variance (ANOVA) was used to determine if oysters differed in size among treatments (shucked or unshucked), replicate piles or bags sampled from piles. Following

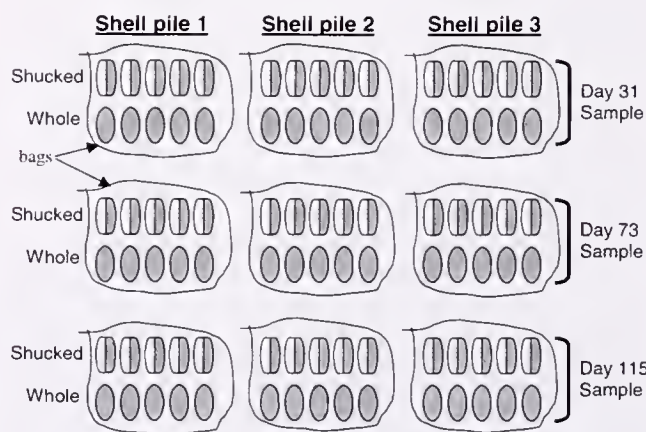


Figure 1. Schematic of the experimental design. $n = 90$ oysters. Forty-five oysters (partially shaded) were shucked and half of the meat removed for analysis at the start of the study. Completely shaded oysters were placed into shell piles as whole live oysters. Five whole and five shucked oysters were sampled from each pile at each sampling interval.

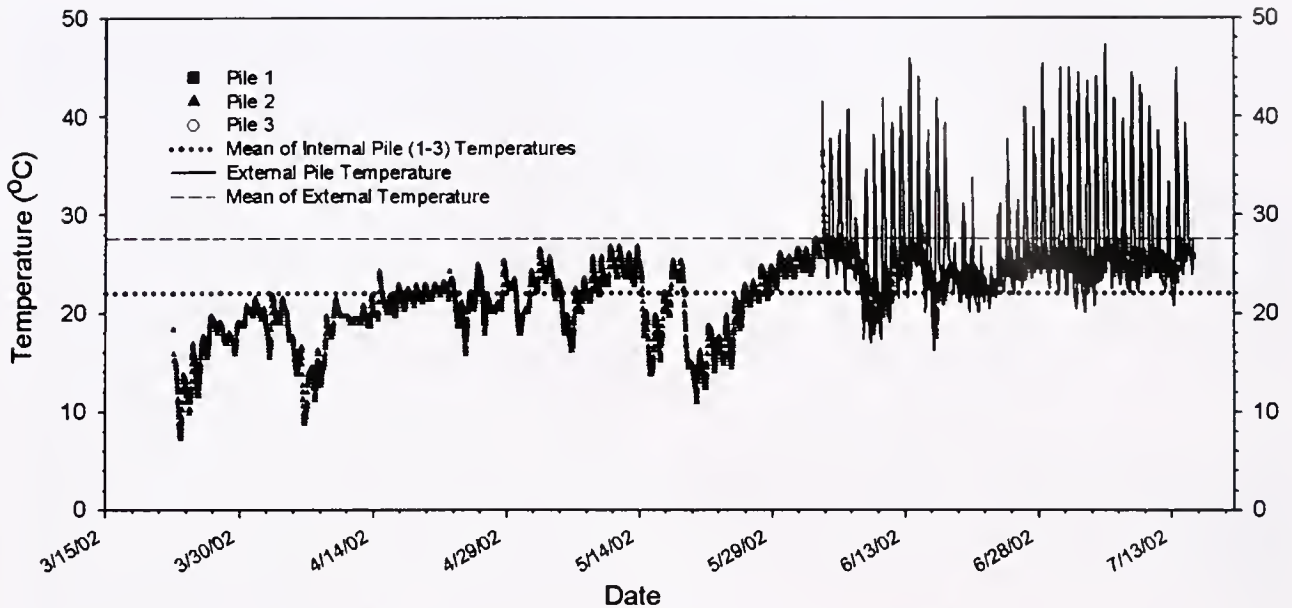


Figure 2. Internal temperatures (symbols) of three shell piles and external temperature (solid line, measured after June 2002) adjacent to the piles measured at 30-min intervals during the study period. Dotted line represents the mean internal temperature of all piles. Dashed line indicates the mean external temperature.

\log_{10} transformation of RFTM counts and assignment of oysters to the various shell piles, mean total body burdens were compared among shell piles. With shell piles as replicates, one-way ANOVAs were also used to compare counts of putative *P. marinus* in RFTM and non-RFTM assays for each treatment on each date. All statistics were carried out using SYSTAT 8.0.

RESULTS

A three-way analysis of variance indicated no significant differences among mean sizes of oysters in bags, piles, or treatments ($P \geq 0.05$ all effects and interactions). Temperature within the piles fluctuated between 6°C and 38°C, with little difference among the piles: means were 21.95°C, 21.83°C, 21.85°C, respectively (Fig. 2). When measured concurrently during the final month of the experiment, temperatures within the three shell piles were cooler on average and less variable than surface temperature (Fig. 2). Temperatures within the piles ranged from 18°C to 37°C with a mean of 25°C, whereas temperatures on the surface ranged from 16°C to 47°C with a mean of 27.5 (Fig. 2). Fluctuations were related to both diurnal (day/night) and climatic changes (e.g., fronts) with external fluctuations always exceeding internal fluctuations. Initial (time zero) RFTM assays indicated 100% prevalence of *P. marinus*. RFTM body burden estimates of *P. marinus* abundance ranged from 180 parasites per oyster to 27.8×10^6 parasites per oyster with a median infection intensity of 95,760 parasites per oyster. Summing RFTM estimates of total body burdens from oysters added to specific piles, and assuming that the portion of tissue added to each pile (i.e., not sampled at time zero) contained half of the total body burden, indicated that 15.8×10^6 parasites were added to pile one, 26.5×10^6 parasites were added to pile two, and 14.0×10^6 parasites were added to pile three for a total of 56.3×10^6 parasites. A one-way ANOVA on \log_{10} -transformed estimates from individual oysters indicated that mean body burdens were not significantly different among piles ($P = 0.513$). Assuming a similar distribution of parasite loads in the 45 un-

shucked oysters, a total of 112.6×10^6 *P. marinus* parasites were present at the start of the experiment in the whole unshucked oysters and about 37.5×10^6 parasites (one-third of the total) were added to each pile in whole unshucked oysters.

Oyster tissues decomposed over time and total parasite abundance declined rapidly (Figs. 3 and 4). After 31 days, only 2 of 15 (13%) shucked oysters contained any tissue (Table 1) and the RFTM assay indicated only 216 parasites remained in tissues from these oysters. No shucked oysters contained any tissue in subsequent samples. In contrast, 12 of 15 (80%) whole oysters contained tissue after 31 days, 10 of 15 (67%) after 73 days, and 2 of 15 (13%) after 115 days (Table 1). The RFTM assay indicated *Perkinsus* abundance dropped 99% by day 31 and declined to only

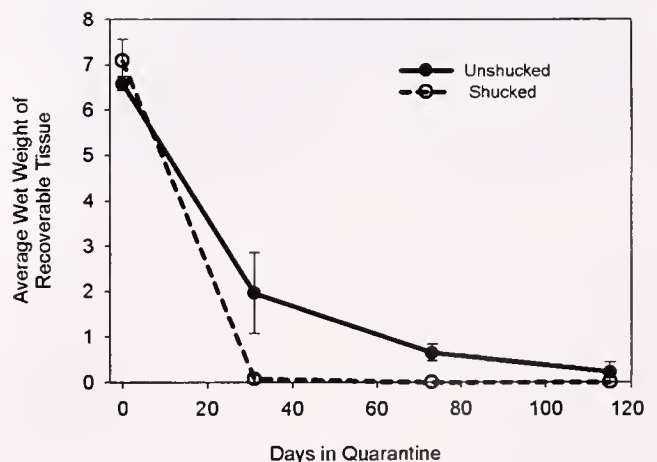


Figure 3. Change in mean wet weight (g) of recoverable tissue in shucked (dashed line) and unshucked (solid line) oysters after 0, 31, 73, and 115 days in shell piles. Error bars represent ± 1 SEM for the three experimental shell piles.

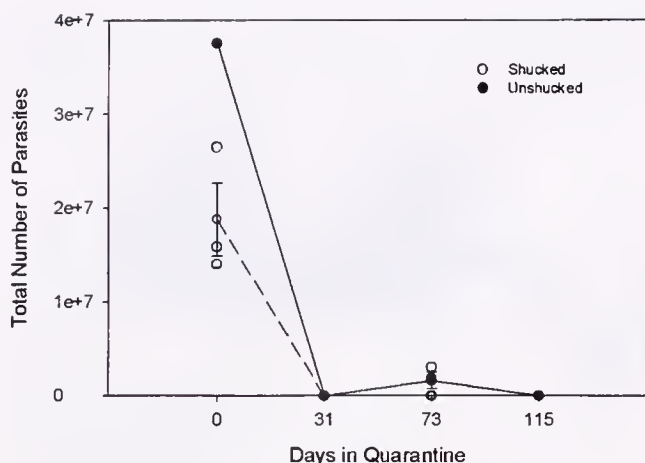


Figure 4. Total estimated number of viable *Perkinsus* cells remaining on shell piles over time in shucked (dashed line) and unshucked (solid line) oysters. Error bars represent ± 1 SEM for the three experimental shell piles.

0.005% of the original abundance by day 115 (Fig. 4). Attempts to isolate and culture *P. marinus* *in vitro* after 2 mo failed.

At time zero, the number of *P. marinus* detected in the non-RFTM assay (18,000 in all oysters) was significantly less than the number detected in the RFTM assay (112.6×10^6 , $P < 0.009$, one-way ANOVA). In all subsequent samples, there were no significant differences between parasites enumerated in RFTM or non-RFTM assays ($P > 0.196$ for all dates). Abundances were unexpectedly higher on day 73 compared with day 31 in both RFTM and non-RFTM assays for whole unshucked oysters due to two oysters with high numbers of apparently unenlarged (5–20 μm) parasites. During RFTM incubation, parasites typically enlarge to 40 μm or more, and observers noted their uncertainty in classifying these unusually small cells as *Perkinsus* while counting them, but small size and microscopic examination was insufficient to disregard them.

DISCUSSION

Considering that *Perkinsus marinus* continues to plague *Crassostrea virginica* throughout most of the oyster's distribution along the Gulf and Atlantic Coasts of the United States, it is only prudent to minimize the spread and proliferation of *P. marinus* whenever possible. A long-standing recommendation about planting oyster seed where *P. marinus* has been problematic is to avoid the use of *P. marinus* infected seed (Andrews & Ray 1988). Though this may seem to be simple common sense, it is not always followed and, to

our knowledge, has never been incorporated into the hygiene of handling cultch before it is planted to collect oyster spat. This study demonstrated that *P. marinus* abundances can be high on fresh cultch, but may decline rapidly (within 1–3 mo) in shell piles. Thus, short-term (1–3 mo) shell pile quarantine may reduce or even eliminate the potential reintroduction and subsequent spread of *P. marinus* when shell is planted to replenish, rebuild, or restore oyster reefs and beds. Nonetheless, several parameters need further exploration.

Not surprisingly, tissue decomposition and parasite decline were faster in shucked animals, most likely because the whole unshucked oysters remained alive for some period in the shell piles. Therefore, whole oysters represent the "worst case scenario" and should be used to establish minimum quarantine duration. Interestingly, except for time zero, the non-RFTM assay revealed concentrations of putative *P. marinus* throughout the study similar to those in the RFTM assay. The lack of significant differences between these samples indicates that the parasites counted in the RFTM assay had likely enlarged in the decomposing tissues prior to RFTM incubation. Unfortunately, the viability of these parasites could not be determined. It is generally assumed that only *Perkinsus* species respond to RFTM incubation by enlarging and developing a thick cell wall that stains blue-black with iodine and is resistant to 2 M NaOH. Furthermore, so far as we are aware, no organisms have been reported from oyster tissues that stain blue-black with Lugol's iodine other than *Perkinsus* spp. It is possible, however, that some of the cells detected with the non-RFTM assay were not *Perkinsus*. Regardless, there were several orders of magnitude fewer parasites on each sampling date after the start of the experiment.

The shell piles used were relatively small (around 100 U.S. bushels) and oysters were buried only 0.5-m deep. This size is reasonable for reef restoration programs in South Carolina (e.g., S.C.O.R.E., www.csc.noaa.gov/scoysters), but is likely much smaller than shell piles produced by commercial shucking operations in other states and used for larger restoration efforts or to replant harvested beds. Decomposition rates are likely to decrease with the size of the shell pile, and we suspect that the abundance of *P. marinus* is closely correlated to decomposition rates of tissue as apparent in this study. It is also likely that decomposition rates are correlated with temperature. Hence, tissue decomposition on shell piles in cooler climates or during cooler portions of the year is apt to be reduced and may increase survival time of *P. marinus* in tissues on the pile.

Thus, though we recommend that shell be quarantined on land for at least a month if not longer, it is clearly apparent that a number of factors should be investigated further. These include temperature and seasonal effects, moisture in the form of humidity or rainfall, and the viability of *P. marinus* in tissues over time. Finally, quarantine impacts for other known or potential oyster pathogens and for human pathogens that are associated with oysters should be investigated similarly.

TABLE 1.

Number of shucked (S) and unshucked (U) oysters with recoverable tissue remaining in shell piles over time.

	Shucked Oysters			Whole Unshucked Oysters		
	Day 31	Day 73	Day 115	Day 31	Day 73	Day 115
Pile 1	1	0	0	4	3	1
Pile 2	0	0	0	4	5	1
Pile 3	1	0	0	4	2	0
Total	2	0	0	12	10	2

ACKNOWLEDGMENTS

The authors thank Dr. Sammy Ray for collecting infected oysters and Jennifer Foster for assistance with RFTM and non-RFTM processing and enumeration of *Perkinsus*. Staff from the SCDNR MRD OFM assisted with shell procurement and shell pile construction. An anonymous reviewer provided comments that

improved the manuscript. Financial support was provided by SCDNR's Marine Resources Division to the Marine Resources Research Institute and by other state funding. This is contract no. 540 of the Marine Resources Research Institute, SCDNR; contri-

bution no. 1382 of the Belle W. Baruch Institute for Marine Sciences and Coastal Research, USC; and contribution no. 2004-3 of the Institute of Marine and Coastal Sciences, Rutgers, The State University of New Jersey.

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EFFECTS OF SALINITY ON GROWTH AND SURVIVAL OF SILVER-LIP PEARL OYSTER, *PINCTADA MAXIMA*, SPAT

JOSEPH J. TAYLOR,² PAUL C. SOUTHGATE^{1*} AND ROBERT A. ROSE³

¹Pearl Oyster Research Group, School of Marine Biology & Aquaculture, James Cook University, Townsville, Queensland 4811, Australia; ²Atlas Pacific, North Fremantle, W.A. 6159, Australia (Current address); ³Pearl Oyster Propagators Pty. Ltd., 4 Daniels St., Ludmilla, N.T. 0820, Australia

ABSTRACT Juvenile silver-lip pearl oysters, *Pinctada maxima* Jameson, were held over a period of 20 days in the following salinities: 45, 40, 34 (ambient), 30, and 25 parts per thousand (‰). There were no significant differences in survival of spat from the different treatments; however, growth was significantly depressed at salinities of 45‰, 40‰, and 25‰. Best growth was recorded at a salinity of 30‰ where spat were significantly larger than those held at ambient salinity. Results indicate that *P. maxima* spat are tolerant of a wide range of salinities; moreover, reduced salinity may be beneficial to growth.

KEY WORDS: salinity, growth, survival, pearl oyster, *Pinctada maxima*

INTRODUCTION

Salinity is a major environmental factor determining the distribution of bivalve molluscs (Hummell 1980, Fuersich 1993). Changes in salinity bring about a broad range of physiologic responses in bivalves and have been shown to influence filtration rate (Riva & Masse 1983, Villiers et al. 1989), oxygen consumption (Bernard 1983), electrolyte balance (Natochin et al. 1981) and the rate of particle transport over the gills (Paparo 1981, Paparo & Dean 1982). Clearly, given these responses, salinity may have a major impact on growth and survival of cultured bivalves.

Salinity tolerances of a number of commercially important bivalves such as the scallops, *Pecten fumatus* (Nell & Gibbs 1986); *Argopecten irradians* (Mercaldo & Rhodes 1982) and *Argopecten ventricosus-circularis* (Singnoret-Brailovsky et al. 1996); oysters, *Ostrea angasi*, *Saccostrea commercialis* (Nell & Gibbs 1986); *O. edulis* (Castagna & Chanley 1973) and *Crassostrea virginica* (Anderson & Anderson 1975); and mussels, *Mytilus edulis planulatus* (Nell & Gibbs 1986) have been determined. Similar research with pearl oysters has been conducted with adult *Pinctada fucata* (Alagarwami & Victor 1976) and *P. fucata martensii* (Kafuku & Ikenoue 1983), and with the larvae of *P. margaritifera* (Doroudi et al. 1999).

The silver-lip (or gold-lip) pearl oyster, *Pinctada maxima*, supports a lucrative cultured pearl industry along Australia's northern coastline. *P. maxima* is found predominantly in oceanic tropical waters of the Indo-Pacific where salinity approximates 35‰ (Gervis & Sims 1992). However, *P. maxima* is often cultured in near-shore estuarine areas that are subject to fluctuating salinity as a result of heavy seasonal rains. For example, pearl oyster cultivation sites in the Northern Territory of Australia experience heavy annual rainfall during the rainy season that may reduce salinity to less than 30‰ for periods of 2 weeks or more (Taylor & Rose, unpublished data, 1993–1996). There is scant information on the effects of environmental fluctuations, such as salinity, on growth and survival of *P. maxima*. This is particularly the case for juveniles that may be more sensitive to environmental change. A prior study reported that increased (to 45‰ and 40‰) and reduced (to 20‰ and 25‰) salinities were an effective means of promoting detachment of *P. maxima* spat (Taylor et al. 1997). Exposure of spat to all four salinities for up to 24 h resulted in significantly

greater detachment than recorded at ambient salinity (34‰), but did not result in mortality. This paper reports on the effects of prolonged exposure to elevated and reduced salinity on growth and survival of *P. maxima* spat.

MATERIALS AND METHODS

After 2 weeks of nursery culture, 45 day-old hatchery-reared *P. maxima* juveniles were returned to the hatchery. They were placed in a 500-L fibreglass tank with a flow-through sea water supply, nominally filtered to 5 µm. Juveniles were fed a mixed micro-algal diet composed of *Chaetoceros muelleri*, *Pavlova salina*, and *Isochrysis* aff. *galbana* (clone T.ISO) for 7 days prior to the start of the experiment. Micro-algae were cultured at 24 °C at a salinity of 34‰ using f/2 medium (Guillard 1972) and subject to a 14:10 h light:dark cycle.

Four treatments and a control, each with 5 replicates, were prepared using gently aerated 1-L plastic aquaria. All seawater used in the trial was nominally filtered to 5 µm. Ambient salinity (control) was 34‰. In two of the treatments, salinity was increased to either 40‰ or 45‰ by adding natural sea salts to seawater. In another two treatments salinity was decreased to either 30‰ or 25‰ by adding rainwater to ambient seawater.

Five *P. maxima* spat were placed into each aquarium. The mean (±s.d.) antero-posterior shell length and dorso-ventral shell height of spat at the start of the experiment were 4.6 ± 0.4 mm and 3.2 ± 0.3 mm, respectively. Spat were fed daily on a mixed micro-algal diet composed of *C. muelleri*, *P. salina*, and T.ISO. The initial ration was 100,000 cells mL⁻¹ day⁻¹ which was gradually increased to 150,000 cells mL⁻¹ day⁻¹ by the end of the experiment. Ten and 20 days after the start of the experiment, surviving spat were counted and measured for shell length and shell height. On day 20, surviving spat were again measured then dried in an oven at 50 °C for 48 hours prior to dry weight determination.

Data Analysis

Shell length, shell height, dry weight, and survival data were compared using ANOVA (Sokal & Rohlf 1981) and means were compared using Fisher's protected least significant difference (PLSD) test from the StatView statistical program, version 4.5, for Macintosh computers (Abacus Concepts, StatView, 1992). Homogeneity of variances was confirmed using Cochran's test (Snedecore & Cochran 1967).

*Corresponding author. E-mail: Paul.Southgate@jcu.edu.au

TABLE 1.

Mean (\pm s.e.) percentage survival of *P. maxima* spat cultured at different salinities for 10 and 20 days. Values in each column with the same superscript are not significantly different ($P < 0.05$).

Salinity (‰)	Percentage of Surviving Spat	
	Day 10	Day 20
45	52.0 \pm 8.0 ^a	44.0 \pm 14.0 ^a
40	76.0 \pm 32.0 ^a	60.0 \pm 20.0 ^a
34	72.0 \pm 10.0 ^a	64.0 \pm 16.0 ^a
30	64.0 \pm 8.0 ^a	44.0 \pm 8.0 ^a
25	68.0 \pm 6.0 ^a	64.0 \pm 8.0 ^a

RESULTS

There were no significant differences ($P > 0.05$) in survival between treatments on either day 10 or day 20 (Table 1). There were, however, significant differences in size between treatments on both occasions. On day 10, spat with the largest mean (\pm s.e.) shell length and shell height were those cultured at salinities of 30‰ and 34‰. At these salinities, there were no significant differences in size ($P > 0.05$); however, both groups of spat were significantly larger ($P < 0.01$) than spat in any other treatment (Fig. 1). By day 20, spat cultured at a salinity of 30‰ had a significantly greater ($P < 0.01$) shell height than spat in all other treatments where shell height did not differ significantly (Fig. 1). Mean shell length of spat cultured at a salinity of 30‰ was significantly larger

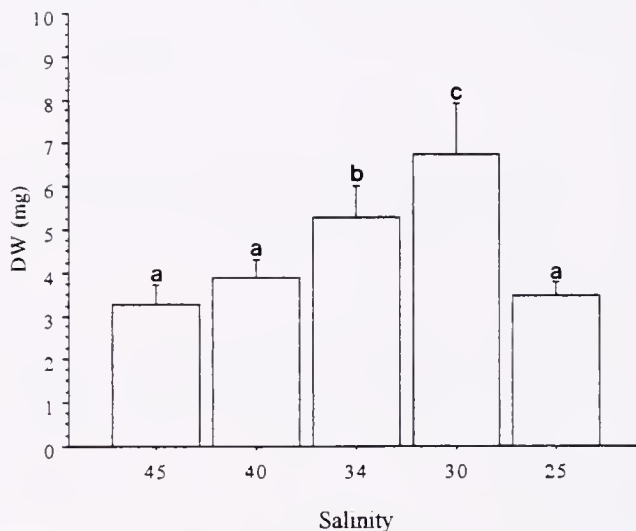


Figure 2. Mean (\pm s.e.) dry weight (DW) of *P. maxima* spat cultured at different salinities (25‰, 30‰, 34‰, 40‰, and 45‰) for 20 days. Means with the same superscript are not significantly different ($P > 0.05$).

($P < 0.01$) than that of spat cultured at salinities of 45‰, 40‰, and 25‰ but did not differ significantly ($P > 0.05$) from that of spat cultured at a salinity of 34‰ (see Fig. 1). The mean shell length of spat held at a salinity of 34‰ did not differ significantly ($P > 0.05$) from those cultured at salinities of 45‰, 40‰, or 25‰. However, dry weight of spat cultured at a salinity of 30‰ was significantly greater ($P < 0.05$) than that of spat held at any other salinity (Fig. 2). Spat cultured at a salinity of 34‰ had a significantly greater ($P < 0.05$) mean dry weight than those cultured at salinities of 45‰, 40‰, or 25‰ that did not differ significantly ($P > 0.05$) from each other.

DISCUSSION

Salinities tested in this study had no significant effect on survival of *P. maxima* spat; however, reducing or increasing salinity significantly affected growth. Reducing salinity to 30‰ significantly improved spat growth, whereas a further reduction to 25‰ or an increase to 40‰ or 45‰ reduced growth. The tolerance of *P. maxima* spat to changes in salinity seems to be greater than that suggested for some oceanic bivalves. The scallop *Pecten fumatus*, for example, has limited tolerance to hypersaline seawater. Within 24 hours of exposure to salinities above 40‰, mantle retraction and gaping have been reported (Heasman et al. 1994) and death occurred in both spat and adult *Pe. fumatus* exposed to such salinities for 48 hours (Heasman et al. 1994, Nell & Gibbs 1986). Rapid decline in the growth rate of *P. maxima* spat cultured at salinities of 30‰ and 25‰ suggests a sharp cut-off point between a reduced salinity that improves growth and one that retards growth. It may be that salinities below 30‰, such as those that occur in parts of northern Australia during the rainy season (Hoppley 1982), may detrimentally effect growth rates of *P. maxima* spat. Such effects may be compounded by the associated run-off from shore and silt and other effluent that may influence coastal pearl oyster culture sites.

Salinity at a pearl oyster (*P. fucata*) culture site in India has been reported to range from approximately 15‰ to 37‰ (Victor, 1983). Such reduction in salinity did not cause mortality of *P. fucata*, which has broad salinity tolerance. Dholakia et al. (1997).

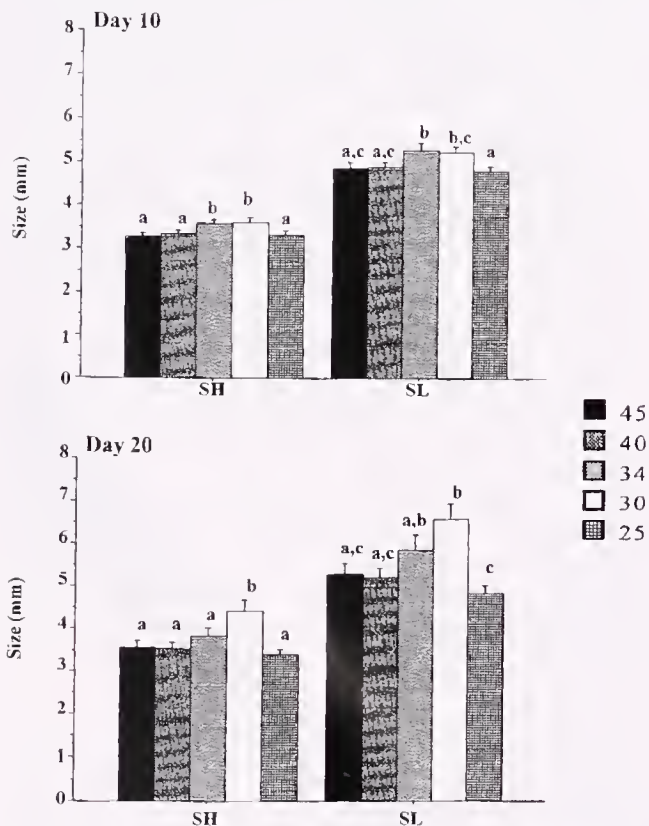


Figure 1. Mean (\pm s.e.) shell height (SH) and shell length (SL) of *P. maxima* spat cultured at different salinities (25‰, 30‰, 34‰, 40‰, and 45‰) for 10 and 20 days. SH and SL values at each time interval with the same superscript are not significantly different ($P > 0.05$).

for example, showed that *P. fucata* is able to survive at salinities ranging from 12‰ to 70‰ when salinity change was gradual (2‰ per day). However, the same study also reported that rapid decrease in salinity, from 55‰ to 15‰, resulted in more than 60% mortality. In a similar study, Jayabhaskaran et al. (1983) reported a relationship between salinity and growth of *P. fucata*, with greater growth rates recorded at "higher" salinity (35‰). Numaguchi and Tanaka (1986) reported that the 'vitality' of *P. fucata martensii* spat decreased sharply at a salinity of 15.2‰ and high mortality resulted when salinity was further reduced to 11.4‰. Numaguchi and Tanaka (1986) concluded that the optimum salinity for culture of *P. fucata martensii* spat was >22.7‰.

The results of this study show that *P. maxima* spat are capable of survival for prolonged periods at salinities ranging from 25‰ to 45‰. They further show a significant influence of salinity on the growth rate of *P. maxima* spat and indicate that the most appropriate salinity range for nursery rearing of *P. maxima* spat is between 30‰ and 34‰. Pearl oysters must reach a minimum shell height before they can be used for pearl production (Pit & Southgate 2003) and this non-productive period is approximately 18 to 24 month for *P. maxima*. It is clearly desirable to minimize this period thereby reducing farm costs and maximizing profitability. Given the influence of salinity on the growth rate of *P. maxima* spat, the results of this study have clear implications for the positioning of nursery culture sites for *P. maxima*.

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WIDESPREAD NULL ALLELES AND POOR CROSS-SPECIES AMPLIFICATION OF MICROSATELLITE DNA LOCI CLONED FROM THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*

DENNIS HEDGEcock,* GANG LI, SOPHIE HUBERT, KATHERINE BUCKLIN AND
VANESSA RIBES

University of Southern California, Department of Biological Sciences, Los Angeles, California 90089

ABSTRACT Non-amplifying, PCR-null alleles are detected at 49 (51%) of 96 microsatellite DNA markers tested for Mendelian segregation in three families of the Pacific oyster *Crassostrea gigas* Thunberg. The average frequency of null alleles among F₁ hybrid grandparents is 0.093. The frequency of null alleles suggests a high level of sequence polymorphism in PCR primer-binding sites and yields a conservative estimate of one single nucleotide polymorphism (SNP) every 82 base pairs. Among 86 markers tested on congeneric species, 83 (96.5%) are likely to be useful markers for the Portuguese oyster *Crassostrea angulata*, 71 (82.6%) for the Kumamoto oyster *C. sikamea*, 31 (36.0%) for the Suminoe oyster *C. ariakensis*, and only 11 (12.8%) for the Eastern oyster *C. virginica*. PCR product-yield and mean numbers of alleles per locus also decline significantly across this series of congeneric species, which separated from the Pacific oyster <1, ~2, ~4, and >5 million years ago, respectively. Decline in cross-specific PCR yield does not depend on microsatellite repeat-motif but is correlated with the frequency of null alleles across loci. The high nucleotide diversity suggested by these observations for the oyster may be a by-product of high fecundity, consistent with G. C. Williams' (1975) Elm-Oyster evolutionary model and experimental evidence for a high mutational load. Microsatellite loci should be identified *de novo* for each species of cupped oyster, and their inheritance should be validated before use in population analyses. Homology of microsatellite loci among related species should be confirmed by sequencing of flanking regions.

KEY WORDS: Pacific oyster, microsatellite DNA, null alleles, cross-specific amplification, nucleotide polymorphism, *Crassostrea gigas*

INTRODUCTION

With the completion or impending completion of genome sequences for the human, fruit fly, nematode, mouse, and other eukaryotic genetic models, much attention is being paid to DNA sequence polymorphism and its potential use in understanding the genetic basis of complex phenotypes, such as disease susceptibility (Zwick et al. 2000). Whereas nucleotide diversity is becoming very well described for model organisms, which are all low-fecundity species (<10₃ – 10₄ eggs per female), little is known about DNA polymorphism in high-fecundity species (>10⁶ eggs per female). We might expect highly fecund species to have high nucleotide diversity, owing to large population sizes, and perhaps higher mutation rates. Indeed, G. C. Williams (1975) argued with his Elm-Oyster Model that highly fecund species with high early mortality (Type-III survivorship) should reproduce sexually, show tremendous variation in individual fitness, and carry a large load of recessive deleterious mutations.

A large load of recessive deleterious mutations has recently been confirmed for the European flat oyster *Ostrea edulis* (Bierne et al. 1998) and the Pacific oyster *Crassostrea gigas* Thunberg (Launey & Hedgecock 2001, Bucklin 2002). Oysters naturally carry dozens of highly deleterious recessive mutations, which explain widespread observations of heterosis for fitness-related traits in bivalve mollusc species, at the whole organism and genetic-marker levels, and distortions of Mendelian segregation ratios at marker loci (Launey & Hedgecock 2004). On a practical level, discovery of genetic load in bivalves suggests that marker inheritance and linkage should be confirmed early in larval development, before selection can substantially distort genotypic proportions. Typing 11-day-old larvae and using double-hybrid crosses to reduce homozygosity by descent and inbreeding depression in mapping families, Hubert & Hedgecock (2004) have produced the first low-density microsatellite DNA marker maps for the Pacific oyster.

Microsatellite DNA markers are short tandem repeats of nucleotide motifs, 2 to 6 base pairs (bp) in length, which are distributed throughout the genome in prokaryotes and eukaryotes (Chambers & MacAvoy, 2000). Because they are highly polymorphic, microsatellites have been widely used as genetic markers for studies of linkage, kinship, and population structure (Goldstein & Schlötterer 1999, Chambers & MacAvoy 2000). Presently, the DNA sequences of 369 microsatellite containing clones from *C. gigas* are deposited in GenBank. Of these, 123 have been developed into PCR-amplifiable markers with confirmed inheritance (Magoulas et al. 1998, Huvet et al. 2000, McGoldrick et al. 2000, Li et al. 2003, Sekino et al. 2003) and 100 have been placed on linkage maps for the Pacific oyster (Hubert & Hedgecock 2004). Here, we present data on polymorphism of 96 of these microsatellite DNA markers and show that there seems to be little or no dependence of polymorphism on repeat-motif or motif complexity.

In developing microsatellite markers for constructing a linkage map of the Pacific oyster, we also uncovered two lines of evidence that nucleotide diversity and rate of sequence evolution in cupped oysters may be extremely high. Because high nucleotide diversity has important implications for future genetic and genomic studies with oysters, we present these findings here. First, we show that there is a high frequency of nonamplifying PCR null alleles, which likely result from polymorphism in the nonrepetitive flanking sequences to which PCR primers are designed to anneal. The inheritance of these null alleles is confirmed in multigenerational families, which were derived from the same population from which the microsatellite markers were cloned. Second, we show a dramatic decay in ability to amplify these markers across a series of four congeneric species that diverged from the Pacific oyster from <1 to >5 million years ago. This decay in cross-species amplification is independent of microsatellite repeat-motif but is correlated with the frequency of null alleles across loci. These observations contrast sharply with reports of success in amplifying microsatellites from very divergent vertebrate taxa (Garza et al. 1995, Pépin et al. 1995, Schlötterer et al. 1991, FitzSimmons et al. 1995, Rico et al.

*Corresponding author. Email: dhedge@usc.edu

1996) and species groups of *Drosophila* flies (Colson et al. 1999, Huttunen & Schlötterer 2002).

MATERIALS AND METHODS

Microsatellite Markers

Inheritance and polymorphism of 96 microsatellite markers was determined for three, multigenerational families that were used to construct linkage maps for the Pacific oyster (see Hubert & Hedgecock 2004). Of the markers that were mapped, 79 come from Li et al. (2003) and 17 come from previous publications (13 listed in Table 1 plus *cmrCgi61*, *cmrCgi141*, *um2Cgi10*, and *um2Cgi48*; Magoulas et al. 1998; Huvet et al. 2000; McGoldrick et al. 2000; see Hubert & Hedgecock 2004). Eighty-six of the 96 markers were further tested in cross-specific PCR amplifications.

Polymorphism and Nonamplifying Null-Allele Frequencies in *C. gigas*

The *C. gigas* used for this study were F₂ or F₃ hybrid parents of the three mapping families, (7 × 6) × (5 × 2), (2 × 5) × (7 × 9), (7 × 9) × (2 × 5), their F₁ grandparents (or, for hybrid line 7 × 6, the F₁ great-grandparents), and other individuals from inbred lines under investigation (Bucklin 2002, Hubert & Hedgecock 2004). These lines were derived from the same Dabob Bay, WA, population, from which the microsatellite DNA clones were obtained (Li et al. 2003). DNA of the F₁ individuals was available from a previous study (Launey & Hedgecock 2001). Whereas families 2 × 5 and 5 × 2 share great grandparents (from inbred lines 92-2 and 89-5, respectively), families 7 × 6 and 7 × 9 are descended from lines 89-7 and 93-7, respectively, and are unrelated.

We typed the eight F₁ grandparents or great-grandparents of these four families to estimate the observed proportion of heterozygous individuals per locus, H_o , and the number of alleles per locus, n_a . Null alleles, which are included in n_a , were identified and confirmed by segregation analysis in mapping families. The

minimum number of independent null alleles per locus (#null in Table 1 and Table 1 of Li et al. 2003) was tallied by tracing allele pedigrees back to inbred great-grandparents (for families 2 × 5 and 5 × 2) or, when allele pedigrees were incomplete (for 7 × 9 and 7 × 6), by assuming that multiple null alleles at a locus were identical by descent. Differences in H_o (arcsine square-root transformed), n_a , and #null among classes of microsatellite motifs were tested by ANOVA. Repeat-motifs were classified as di-, tri-, or tetra-nucleotide repeats or alternatively cross-classified into categories of motif-complexity (simple or compound vs. pure or interrupted by nonmotif nucleotides; following Chambers & MacAvoy 2000, with compound and complex motifs combined).

Cross-species Amplifications

For cross-species comparison, we obtained DNA from five or six individuals of three other Asian species and the American species of *Crassostrea*, as described by Li et al. (2003). For each of 86 markers, PCR amplifications were done simultaneously for the five species, using a single PCR reaction mix, followed by a single acrylamide gel separation and fluorescent scan. PCR product was quantified by peak optical density per pixel (OD) in bands of similar size and appearance as bands observed in *C. gigas* on the same gel, using FMBIO software (Hitachi Genetic Systems). Owing to variable DNA template concentrations and a significant positive regression, within *C. gigas*, of OD on initial DNA concentration ($F_{1,1135} = 9.95$, $P = 0.0017$), we used a natural log transformation, $adjOD = \ln[(OD + 10)/C]$, where C is template DNA concentration. Regression of $adjOD$, on C was not significant ($F_{1,1135} = 1.02$, $P = 0.31$).

Variation in $adjOD$ was analyzed with linear models (GLM procedure of SAS version 7, SAS Institute, Inc., Cary, NC) of completely randomized block design, with 5 species × 3 repeat-motif or 4 cross-classified categories of motif-complexity × 2 different observers working with different DNA extractions (blocks). There were 3051 observations in these ANOVAs for repeat motif and 2593 observations for motif-complexity categories. Significance of block and main effects was tested by the within-block variance of species-motif combinations, using Type III sums of squares. Relationship between null-allele counts within *C. gigas* and ability to amplify markers from congeneric species was determined by stepwise regression, with $adjOD$ dependent on species and null-allele count.

Allelic diversity per locus was estimated by counting the number of unique bands (n_a) observed per marker, per species (over all markers and individuals, $n = 380$). Six 7 × 6 F₃ hybrid individuals were included in this analysis as an additional comparison. Analysis of these data must account for varying numbers of individuals successfully amplified, per species and PCR reaction (n_r), and an expected, positive correlation between n_a and n_r . The number of individuals amplified was used as a covariate in a 2-factor ANCOVA of species and repeat motif (6 × 3). Least-squares estimates of the n_a for each species, adjusted to a constant $n_r = 5$, were obtained with estimate statements in PROC GLM.

A qualitative and partially subjective assessment was made of whether each marker worked sufficiently well in another species to advise its use as a genetic marker for that species. A marker was judged likely to work for another species if it amplified from at least four of the five or six individuals surveyed and it produced bands of sufficient strength for genotypes to be reliably determined. These assessments are listed under the "cross species"

TABLE 1.

Cross-species amplification success and number of null alleles for 13, previously published, Pacific oyster microsatellite loci.

Locus ^a	<i>C. angulata</i>	<i>C. sikamca</i>	<i>C. ariakensis</i>	<i>C. virginica</i>	#null in <i>C. gigas</i> ^b
<i>cmrCgi1</i> ^c	+	+	—	—	0
<i>cmrCgi3</i> ^c	+	+	—	—	0
<i>cmrCgi5</i> ^c	+	+	+	—	1
<i>imbCgi44</i> ^d	+	+	—	—	3
<i>imbCgi49</i> ^d	+	—	—	—	1
<i>imbCgi108</i> ^d	+	—	—	—	0
<i>ucdCgi1</i> ^c	+	+	+	+	0
<i>ucdCgi2</i> ^c	+	+	—	+	0
<i>ucdCgi3</i> ^c	+	—	—	—	0
<i>ucdCgi6</i> ^c	+	—	—	—	0
<i>ucdCgi14</i> ^c	+	+	—	—	1
<i>ucdCgi18</i> ^c	?	+	+	+	2
<i>ucdCgi28</i> ^c	+	+	+	—	1

^a Loci names modified slightly from the original published name to conform to conventional nomenclature.

^b Number of independent null-alleles in F₁ hybrid grand- or great grandparents

^c McGoldrick et al. 2000; Launey & Hedgecock 2001

^d Magoulas et al. 1998

^e +, amplification; —, insufficient amplification; ?, slight amplification

column of Table 1 of Li et al. (2003), for the 79 loci presented there, and are summarized here in Table 1, for previously published markers, as "+," for amplification, "-", for insufficient amplification, and "?," for slight amplification.

RESULTS

Heterozygosity and Null-allele Frequencies

The 96 microsatellite markers used for determination of polymorphism in *C. gigas* comprise 58 di-, 22 tri- and 16 tetra-nucleotide repeat-motif. Alternatively they can be classified into 4 crossed categories of motif-complexity: 41 are simple-pure, 23, simple-interrupted, 11, compound-pure and 21, compound-interrupted (Table 2). Interrupted repeat arrays comprise 45.8% (44/96) of the loci tested. These markers vary widely in degree of polymorphism, with numbers of alleles (n_a), ranging from 2 to 10 (mean, 5.77 ± 0.14) and observed heterozygosity (H_o), ranging from 0.125 to 1.0 (mean, 0.748 ± 0.047 ; see Table 2). Neither measure of polymorphism, however, varies significantly by repeat-motif (di-, tri-, and tetra-nucleotide motifs; for n_a , $F_{2,93} = 0.90$, $P = 0.41$ and for H_o , $F_{2,93} = 0.81$, $P = 0.45$) or motif-complexity category (simple-pure, compound-pure, simple-interrupted, compound-interrupted; for n_a , $F_{2,93} = 1.77$, $P = 0.16$, and for H_o , $F_{2,93} = 1.33$, $P = 0.27$) in our sample of F_1 hybrids.

Non-amplifying PCR-null alleles are found at 49 (51%) of the 96 loci. Minimum counts of independent PCR-null alleles per locus, in the F_1 grandparents or great grandparents of the mapping families, range from 0 to 4 and sum, over all loci, to 75 (Fig. 1). The average number of independent alleles per locus is 8.6, and the average frequency of null alleles is 0.093. Neither null-allele frequency nor null-allele count (see Table 2) varies among repeat-motifs ($F_{2,93} = 1.53$, $P = 0.22$) or motif-complexity categories ($F_{3,92} = 0.83$, $P = 0.48$).

Cross-species Amplifications

Among 86 markers tested on congeneric species, 83 (96.5%) are likely to be useful for *C. angulata*, 71 (82.6%) for *C. sikamea*, 31 (36.0%) for *C. ariakensis*, and only 11 (12.8%) for *C. virginica*. These qualitative observations of amplification are supported by a

TABLE 2.

Observed heterozygosity, numbers of alleles, and numbers of independent null alleles, by (A) repeat motif and (B) motif-complexity categories, for 96 microsatellite markers in *C. gigas*.

A. Repeat number

Motif (number of loci)	$H_o \pm SE$	$n_a \pm SE$	$\#null \pm SE$
Di-nucleotide (58)	0.769 ± 0.028	5.93 ± 0.26	0.948 ± 0.128
Tri-nucleotide (22)	0.704 ± 0.046	5.27 ± 0.42	0.545 ± 0.207
Tetra-nucleotide (16)	0.729 ± 0.054	5.88 ± 0.50	0.688 ± 0.243
Overall (96)	0.748 ± 0.022	5.77 ± 0.20	0.812 ± 0.100

B. Motif complexity

Simple, pure (41)	0.742 ± 0.033	5.54 ± 0.31	0.683 ± 0.153
Compound, pure (11)	0.758 ± 0.064	6.54 ± 0.59	1.182 ± 0.295
Simple, interrupted (23)	0.689 ± 0.045	5.30 ± 0.41	0.783 ± 0.204
Compound, interrupted (21)	0.817 ± 0.047	6.33 ± 0.43	0.905 ± 0.214
Overall (96)	0.748 ± 0.022	5.77 ± 0.20	0.812 ± 0.100

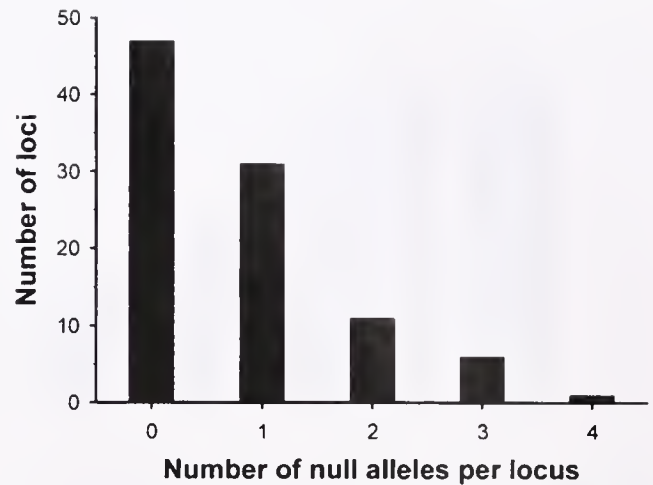


Figure 1. Distribution of 96 microsatellite markers, according to the number of potentially independent null alleles in 4 lineages of Pacific oysters. Forty-nine markers (51%) have at least 1 null allele, and the total number of potentially independent null alleles is 75.

quantitative analysis of PCR yields. Variance in amount of PCR product, as measured by peak optical density, is corrected for differences in initial concentration of template DNA (adjusted OD). Variance in $adjOD$ is analyzed by 2-way, randomized, complete-block linear models with fixed effects. Both models are highly significant, the first accounting for 41.2% of variance in adjusted OD and the second accounting for 41.3% of variance. In both models, the block effect (different observers working with different DNA extractions) is nonsignificant. Species, on the other hand, has a highly significant effect on adjusted OD in both analyses ($F_{4,14} = 81.71$, $P < 0.0001$, for the repeat-motif analysis, and $F_{4,19} = 36.13$, $P < 0.0001$, for the motif-complexity analysis). Repeat-motif has only a mildly significant effect on adjusted OD ($F_{2,14} = 3.99$, $P < 0.043$), motif-complexity has no effect on adjusted OD, and species-by-motif interaction terms are nonsignificant. Mean adjusted OD declines from 12.6 in the focal species, *C. gigas*, and 12.4 in its nearest relative, *C. angulata*, which are not significantly different, to 10.9, 8.1, and 6.7 in the nonfocal species *C. sikamea*, *C. ariakensis*, and *C. virginica*, which are significantly different from *C. gigas* and *C. angulata* and from each other (Fig. 2).

Regression of adjusted OD on species and null-allele count per locus ($\#null$) in *C. gigas* is significant for both variables. Species explains 35% of variance in the adjusted OD, whereas null-allele count per locus, though significant, accounts for only 0.3% of variance ($adjOD = 14.772 - [1.481 \cdot species] - [0.210 \cdot \#null]$; $F_{1,3012} = 1647.6$ for *species*, $P < 0.0001$ and $F_{1,3012} = 13.04$, $P < 0.0003$ for $\#null$).

Variance in allelic diversity is analyzed with 2-way linear models, in which the main effects are species (six, including an F_3 hybrid group of *C. gigas*) and either repeat-motif or motif-complexity categories. The dependent variable, n_a , is standardized by the covariate n_i , the number of individuals amplified. Both models are significant, accounting for 43.8% of variance in the analysis of species and repeat motifs and 41.6% in the analysis of species and motif-complexity categories. In both models, the covariate is highly significant ($F_{1,394} = 61.4$, $P < 0.0001$, and $F_{1,388} = 43.74$, $P < 0.0001$, respectively) and positively related to allelic diversity (slopes of 0.43 and 0.37, respectively), as expected. Spe-

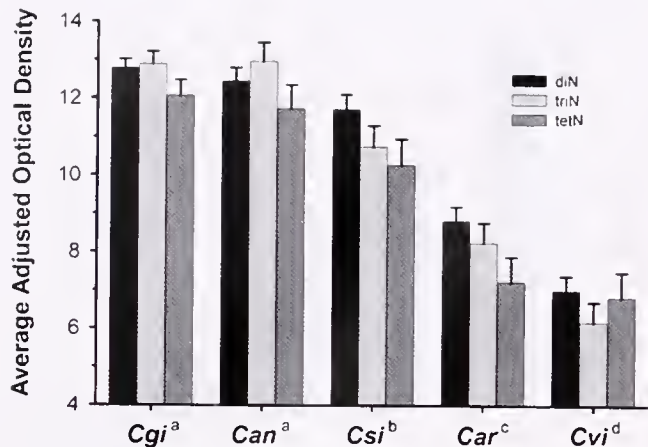


Figure 2. Average adjusted optical density (least-squares means and standard errors) of amplified microsatellite DNA markers with di-, tri-, and tetra-nucleotide repeat-motifs, among 5 species of *Crassostrea*. *C. gigas* (Cgi, $n = 1137$), *C. angulata* (Can, $n = 516$), *C. sikamea* (Csi, $n = 430$), *C. ariakensis* (Car, $n = 484$), and *C. virginica* (Cvi, $n = 484$). Superscripts on species names indicate significant differences; bars give the upper 95% confidence limits on least-squares means. diN, di-nucleotide repeat; triN, trinucleotide repeat; tetN, tetra-nucleotide repeat.

cies is also highly significant in both models ($F_{5, 394} = 16.08$, $P < 0.0001$, and $F_{5, 388} = 17.43$, $P < 0.0001$, respectively). Repeat-motif is significant ($F_{2, 394} = 4.21$, $P = 0.016$), whereas motif complexity is barely significant ($F_{3, 388} = 2.78$, $P = 0.041$). The interaction terms are not significant in these analyses. Mean number of alleles, adjusted to a common sample size of 5 individuals, declines from 4.54 and 4.58 in the *C. gigas* and *C. angulata* (not significantly different), to 3.42, 2.64, and 2.09 in *C. sikamea*, *C. ariakensis*, and *C. virginica*, respectively (Fig. 3). The 7×6 F_3 hybrid *C. gigas* have 4.27 alleles on average, fewer but not significantly fewer than noninbred *C. gigas* or *C. angulata*. Mean n_a for trinucleotides, 3.13, is significantly less than the mean

for tetra-nucleotide repeats (3.85) or di-nucleotide repeats (3.51), respectively.

DISCUSSION

Polymorphism in *C. gigas*

The diversity of repeat motif and motif-complexity of markers used in this study should help reduce bias in polymorphism or distribution across the oyster genome (Tóth et al. 2000, Katti et al. 2001). Interruptive mutations, which occur in almost half of the microsatellite loci in *C. gigas* (see Table 2), are hypothesized to be the first step in the "death" of microsatellites because they prevent slipped-strand mispairing and stabilize the repeat (Taylor et al. 1999). This scenario is not supported here, because neither heterozygosity nor number of alleles per locus varies among repeat-motif or motif-complexity categories. However, the power of this test is uncertain, because we measure heterozygosity and allele diversity in only four pairs of sibling F_1 hybrids. Nevertheless, we do compare a large number of loci in each repeat-motif category. Ideally, one would like to base comparisons of polymorphism among microsatellite motifs on large samples from natural populations, but the widespread occurrence of null alleles confounds estimation of heterozygosity and allelic diversity in natural populations.

Null Alleles at Microsatellite DNA Markers

Null alleles at allozyme loci have been detected in experimental crosses of several bivalve species (see references in Gaffney 1994). Gaffney (1994) reported null alleles at 10 of 11 allozyme loci in the coot clam *Mulinia lateralis*, with an average frequency per locus of 0.04. Since the advent of PCR, nonamplifying null alleles at DNA markers have been reported in the American oyster, the Pacific oyster, and the geoduck clam (Hu & Foltz 1996, Launey & Hedgecock 1999, 2001, McGoldrick et al. 2000, Vadopalas & Bentzen. 2000, Reece et al. 2001). Among 47 family tests of Mendelian transmission of microsatellites in Pacific oyster families, 17% of parental alleles were nonamplifying (McGoldrick et al. 2000). Similarly, of 94 segregation ratios tested in seven families by Launey & Hedgecock (2001), 15 showed unexpected progeny phenotypes that were best explained by null alleles. More recently, Sekino et al. (2003) report population data for nine microsatellite DNA markers in the Pacific oyster; of eight loci that are polymorphic, four show a deficiency of heterozygotes that is significant at the nominal 5% level. The mean frequency of null alleles at these four loci is 0.111.

Of 96 microsatellite DNA markers assayed in this study, 49 (51%) have at least one nonamplifying null allele in the three families examined (see Fig. 1). This is especially surprising, because these families were derived from the same natural population from which the microsatellite libraries were originally cloned. The average frequency of null alleles for oyster microsatellite loci, 0.093, is twice as high as the frequency of allozyme null alleles. If we make the simple assumption that each PCR-null allele is caused by a single nucleotide polymorphism (SNP) in a primer-binding site, then, given 3997 nucleotide base pairs in the PCR primers for 96 loci, we infer a SNP density of $49/3997 = 0.0123$, one SNP every 82 base pairs. This is likely to be an underestimate, because not all nucleotides in primer binding sites are essential to primer binding. An estimate based on the assumption that the 75 null alleles in independent lineages are different, 0.0188, is signifi-

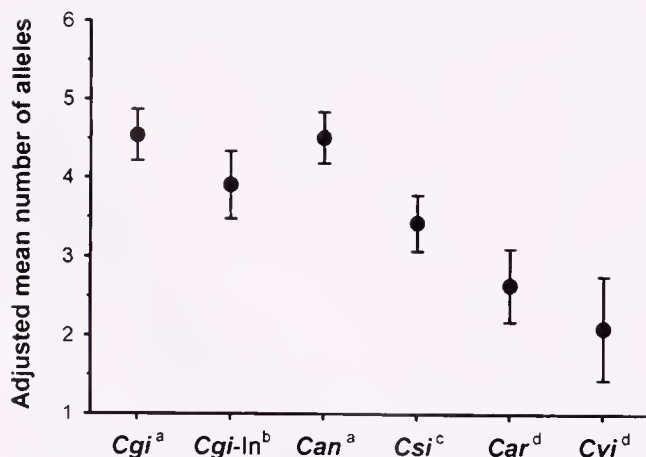


Figure 3. Mean number of alleles, standardized to 5 individuals, for microsatellite DNA markers, amplified from 5 species of *Crassostrea* and an F_3 hybrid group of *C. gigas* (Cgi-In): *C. gigas* (Cgi, $n = 85$; Cgi-In, $n = 52$), *C. angulata* (Can, $n = 85$), *C. sikamea* (Csi, $n = 78$), *C. ariakensis* (Car, $n = 50$), and *C. virginica* (Cvi, $n = 30$). Superscripts on species names show significant differences. Bars show 95% confidence intervals on least-squares means.

cantly greater, but this may be an overestimate if many of the nulls segregating among inbred lines are allelic. The frequency of non-amplifying null alleles at microsatellite DNA loci in this oyster population suggests high nucleotide polymorphism in *C. gigas*.

In population studies, PCR-null alleles produce an excess of homozygotes relative to Hardy-Weinberg (random mating) equilibrium genotypic proportions. Although PCR-null alleles may be visualized by redesigning primers to more conservative parts of the flanking region (Jones et al. 1998), primer redesign does not guarantee that all PCR-null alleles will be eliminated from all populations of potential interest. It will be necessary to exercise caution in applying microsatellite markers to studies of natural bivalve populations.

Decline in Cross-species Amplification of Microsatellite Markers

The congeneric species used in this study are thought to have diverged from the Pacific oyster from <1 to >5 million years ago (Mya). Divergence times, however, must be inferred largely from molecular differences, without benefit of a fossil record or other means for calibrating rates of molecular evolution.

The Portuguese oyster *C. angulata* is the closest relative of the focal species *C. gigas*. Indeed, the Portuguese oyster is believed to be synonymous with the Pacific oyster, based on similarity of allozymes, indistinguishable larval and adult shells, complete cross-fertility, and normal meiosis in hybrids (see Boudry et al. 1998, Ó Foighil et al. 1998, and references therein). Mitochondrial DNA analysis implicates Taiwan as the source of introduction of this oyster to Portugal, likely by 16th century Portuguese traders (Boudry et al. 1998). An average 2.3% difference in mitochondrial cytochrome oxidase I (COI) nucleotide sequence suggests, however, that the Taiwanese and Japanese populations of this species may have diverged several hundred thousand years ago (Ó Foighil et al. 1998). The next closest relative of *C. gigas* is the Kumamoto oyster *C. sikamea*, which diverged perhaps 1.4–1.8 Mya, based on mitochondrial 16S rDNA sequences and allozymes (Banks et al. 1994). This divergence may also be estimated as ~2.3 Mya, based on the 9.3% average nucleotide difference for the COI sequences determined by Ó Foighil et al. (1998) and assuming a 2% per million years rate of evolution. In the same manner, an average of 14.5% nucleotide difference between the COI sequences of *C. gigas* and *C. ariakensis* suggests a divergence of ~3.6 Mya, whereas the separation of the American oyster *C. virginica* from the Asian *Crassostrea* clade (*C. gigas*, *C. ariakensis*, and *C. belcheri*) might have occurred more than 5 Mya. The latter seems to be an underestimate based on Littlewood's (1994) finding that sequences for 28S rDNA suggest that *C. virginica* is a sister group, along with *Saccostrea commercialis*, to the Asian *Crassostrea* clade.

Thus, ability to amplify microsatellite markers developed for the Pacific oyster from the DNA of congeneric *Crassostrea* species declines precipitously over an evolutionary time span of only 5–10 million years (see Fig. 2). Fewer than one out of eight Pacific oyster microsatellite markers are likely to be useful for the American oyster. Of course, we may have underestimated PCR success in nonfocal species because of 3 limitations in the survey. First, only a single PCR reaction and gel was run for each primer set, though on multiple individuals and always with the same *C. gigas* controls. Second, comparisons were performed under the PCR condition optimized for the focal species. Third, homology of PCR products across congeneric species was assumed for fragments in

the same size range as those observed in *C. gigas*, without being confirmed by sequencing. Despite these limitations, we observe a clear signal of PCR decay, perhaps owing to the large number of comparisons, 86 markers, and more than 3000 observations.

The slight but highly significant, negative correlation between null-allele frequency within *C. gigas* and PCR product yield from closely related species implies that failure of cross-species amplification results from evolution of flanking sequences. Fixation of nucleotide substitutions and insertions/deletions causes widespread PCR-null alleles within *C. gigas*, and this same process, over evolutionary time scales, results in loss of ability to amplify homologous loci from related species.

Decline in the ability to amplify Pacific oyster microsatellites from congeneric species is paralleled by a decline in allelic diversity for those markers that do amplify (Fig. 3). The validity of this trend is supported by the observation that an F₃ hybrid family of Pacific oysters has fewer alleles than the various F₁ hybrid or first generation inbred Pacific oysters or the unrelated individuals of *C. angulata* surveyed. The decline in allelic diversity across the congeneric species surveyed is consistent with ascertainment bias, the expectation that, owing to a relationship between allele size and polymorphism, loci selected to be polymorphic in a focal species are likely to be less polymorphic in other species (Ellegren et al. 1995; Goldstein & Pollock 1997). For the limited data in Table 1 of Li et al. (2003), allele size seems to be uncorrelated with either heterozygosity or allele-diversity, leaving the cause of ascertainment bias uncertain.

Homologous microsatellite loci have been successfully amplified with the same PCR primers from very divergent animal taxa—humans and chimpanzees (4–6 Mya), cow and goat (14–17 Mya), cetaceans (35–40 Mya), marine and freshwater turtles (300 Mya), and fish (470 Mya), (Schlötterer et al. 1991; Garza et al. 1995; Pépin et al. 1995; FitzSimmons et al. 1995; Rico et al. 1996). These studies suggest that microsatellite flanking regions are well conserved in vertebrate taxa, at least in selected cases.

Studies of cross-specific amplification of microsatellites in *Drosophila* provide invertebrate comparisons. Again, many studies have selected markers that could be amplified in related *Drosophila* species to reconstruct phylogenies (e.g., Noor et al. 2001, Noor M. personal communication). Two studies, however, seem to have tested cross-specific amplifications for a large, randomly selected set of microsatellite loci. Colson et al. (1999) report that 86 (80.4%) of 107 microsatellite loci characterized from *D. melanogaster* can be amplified from *D. simulans* and *D. sechellia*. Based on the nucleotide divergence of *Adh* sequences and a rate of evolution calibrated for Hawaiian species, which are endemic to islands of known age, the latter two species are estimated to have diverged from the focal species about 2.3 ± 0.65 Mya (Russo et al. 1995). These authors point out that this estimate has large standard errors and may not be inconsistent with previous estimates of 2–5 Mya. The results of Colson et al. (1999) are thus comparable with the 82.6% amplification success of *C. gigas* microsatellites from *C. sikamea*, which are believed to have diverged ~2 Mya. However, in a study of the *Drosophila virilis* group, Huttunen & Schlötterer (2002) report that, of 42 microsatellite markers developed from *Drosophila virilis*, 34 (81%) and 32 (76%) can be amplified from *D. montana* and *D. flavomontana*, respectively. Based, again, on divergence of *Adh* sequences and the Hawaiian calibration of evolutionary rate, Nurminsky et al. (1996) estimate that the *virilis* and *montana* clades diverged 9.0 ± 0.07 Mya. The success of cross-specific amplification in the *virilis* group is

clearly much higher than what we observe for the *C. gigas* versus *C. ariakensis* (36.0%) or *C. gigas* versus *C. virginica* (12.8%) comparisons. Compared with vertebrates or *Drosophila*, then, cross-specific amplification of Pacific oyster, microsatellite DNA markers show an unusually precipitous decline across a series of closely to progressively more distantly related species.

Nucleotide Diversity and High Fecundity

The evidence for high nucleotide polymorphism in the Pacific oyster is interesting in light of recent experimental evidence for a high mutational load in this species (Launey & Hedgecock 2001, Bucklin 2002). High mutational load, nucleotide diversity, and rapid sequence evolution may all be by-products of sexual reproduction in highly fecund organisms having very high rates of mortality in early life stages (Williams 1975). Female Pacific oysters routinely spawn tens of millions of eggs per year, with an upper limit of about 100 million per year (Galtsoff 1964). Species with very high fecundity likely generate more mutations than low fecundity species, owing to the large number of cell divisions required to produce millions or billions of gametes (cf. similar to the argument for male-driven evolution in humans, Li et al. 2002). An association of high mutation rate with high fecundity was previously proposed to explain the distribution of mitochondrial DNA variant haplotypes in the Pacific oyster (Beckenbach 1994). An extremely variable position of *C. virginica* in a bivalve phylogeny based on 18S rDNA was also attributed to a high substitution rate of this species (Steiner & Muller 1996). Whether the mutation rate per-cell-division, as well as the number of cell divisions, is higher in oysters than in low-fecundity species is so far unknown. The high rate of somatic cell aneuploidy in bivalve molluscs, which

correlates with reduced individual growth and probably fitness in the Pacific oyster (Leitão et al. 2001), suggests that important cellular and molecular mechanisms may be more labile in these animals than in vertebrates.

CONCLUSION

The evidence obtained from developing and mapping microsatellite DNA markers suggests that nucleotide sequences in the Pacific oyster are mutating and diverging rapidly. This nucleotide diversity has a dramatic impact on the population genetics and evolution of microsatellite markers. Nucleotide substitutions and insertions/deletions in microsatellite flanking regions produce a high frequency of null-alleles, even within the source population from which microsatellites were cloned, and contribute to failure in amplification of microsatellite markers from closely related, congeneric species. As a result, microsatellite loci should probably be identified *de novo* for each new species and validated with progeny testing before use in population analyses. Homology of microsatellite loci among related species should be confirmed by sequencing before use in phylogenetic analysis.

ACKNOWLEDGMENTS

We dedicate this manuscript to the memory of our friend and colleague, Will Borgeson, who reared and cared for oyster stocks used in this study. The authors thank Drs. P. Boudry, IFREMER, La Tremblade, France, and S. K. Allen, Jr. and K. Reece, Virginia Institute of Marine Science, for providing oyster samples. This research was supported by USDA National Research Initiative Competitive Grants Program (agreement number 99-35205-8260).

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A COMPARISON OF DREDGE AND PATENT TONGS FOR ESTIMATION OF OYSTER POPULATIONS

ROGER MANN,^{1,*} MELISSA SOUTHWORTH,¹ JULIANA M. HARDING¹ AND JAMES WESSON²

¹Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062;

²Virginia Marine Resources Commission, P.O. Box 756, Newport News, VA 23607-0756

ABSTRACT Exploited oyster stocks on public grounds in Virginia waters are subject to regular surveys effected using a traditional oyster dredge and, more recently, patent tongs. Dredges provide semiquantitative data, have been used with consistency over extended periods (decades), and provide data on population trends. Surveys with patent tongs provide absolute quantification (number of individuals per unit area) of oyster stocks but are more labor intensive. Absolute quantification of dredge data is difficult in that dredges accumulate organisms as they move over the bottom, may not sample with constancy throughout a single dredge haul, and may fill before completion of the haul thereby providing biased sampling. Selectivity of dredges versus patent tongs with respect to oyster demographics has not been rigorously examined. The objective of this study is to compare demographic oyster data collected at the same sites in the same years from both gear types. Data for the study were taken from 1993 to 2001 surveys conducted in the James River, Virginia, by the Virginia Institute of Marine Science and the Virginia Marine Resources Commission wherein the same stations were sampled by both techniques. Dredge surveys give data in oysters per bushel and assume no selective retention of live oysters with respect to shell substrate by the dredge. Patent tong surveys provide data as per tong estimates of oysters by size class and shell by volume. The hydraulically operated, 1-m square tong used in VMRC/VIMS surveys is designed to sample on and below the reef surface and include elements of buried shell that are probably not well sampled by a dredge, although the sampling ensures collection of all oysters within the tong mouth. Oysters collected by both gear types were classified as small (25–75 mm) or market (>75 mm SL) for comparisons across methods. Shell volumes collected in patent tong surveys were standardized to bushel increments assuming 35.28 L of shell per bushel. The summary plots of mean values from 1993 to 2001 and 1998 to 2001 illustrate differences related to sampling gear. More shell per unit oyster (lower bushel counts) are observed in a patent tong sample. The appropriate model for attempting to fit a predictive line is open to debate, and will be influenced by patent tong penetration as determined by the degree of consolidation of the underlying substrate. The available data do not strongly support the ability to predict a relationship between dredge and patent tong population estimates at this time.

KEY WORDS: *Crassostrea virginica*, Eastern oyster, recruitment, survey methods, Chesapeake Bay

INTRODUCTION

The Chesapeake 2000 agreement (Chesapeake Bay Program, 2000) established a 10-fold increase in the Chesapeake Bay oyster population by 2010 as one of the principal goals of Bay restoration. The baseline for this goal is the population biomass that existed at the beginning of 1994, as assessed by surveys in fall 1993. A collaborative research effort between researchers in Maryland and Virginia was performed between 2000 and 2003 to develop the baseline value. Historical data in both states was collected in support of fishery management although differences existed in details of the assessment methods preventing a singular approach to stock estimation from the accumulated data sets. Dredge surveys have been used for oyster stock assessment in Virginia for over 50 years. The Virginia Institute of Marine Science (VIMS) began annual dredge surveys at a limited number of sites in Virginia's subestuaries in 1946 and this sampling program has continued through the present with recent dredge surveys conducted in collaboration with the Virginia Marine Resources Commission (VMRC) Shellfish Replenishment Program. More recently, dredge stock assessments have been supplemented by annual patent tong surveys based on a stratified random sampling design. Patent tong sampling began in the James River in 1993 and has since expanded to include the Piankatank River (1998 to present), Great Wicomico River (1998 to present), Rappahannock River (1993, 1995–1997, 1998 to present), Tangier Sound (2001 to present) and Pocomoke

Sound (2002 to present). Here we focus exclusively on data from the James River, Virginia.

Dredge data is semiquantitative. The data exist as numbers of oysters per bushel, not bushels per unit area. Patent tong data are absolute density data (numbers of oysters per unit area). Given both the extensive historical and spatial coverage of dredge assessment, the question was posed as to the possibility of developing a conversion function relating dredge to patent tong data, thus allowing hindcasting of absolute densities of oyster populations using historical dredge data sets. This, in turn would facilitate description of historical oyster populations including estimation of the 1994 baseline for the previously described 10-fold increase goal. In this note we compare oyster density estimates from both dredge and patent tong sampling for a limited suite of observations, 1993 to 2001 surveys conducted in the James River, where both sampling approaches were used on the same populations.

MATERIALS AND METHODS

Intensive sampling of the areas within the James River, Virginia that are actively fished for oysters (Fig. 1) is completed each fall as part of a collaborative program between VMRC and VIMS. Patent tong surveys use a stratified random sampling design with historical reefs (Baylor 1894, Haven et al. 1981) forming the basis of strata delineation. Individual samples were collected by a hydraulic patent tong with a one square meter sample capability. The patent tong used in VMRC/VIMS surveys is designed to sample on and below the reef surface, and include elements of buried shell

*Corresponding author. E-mail: rmann@vims.edu

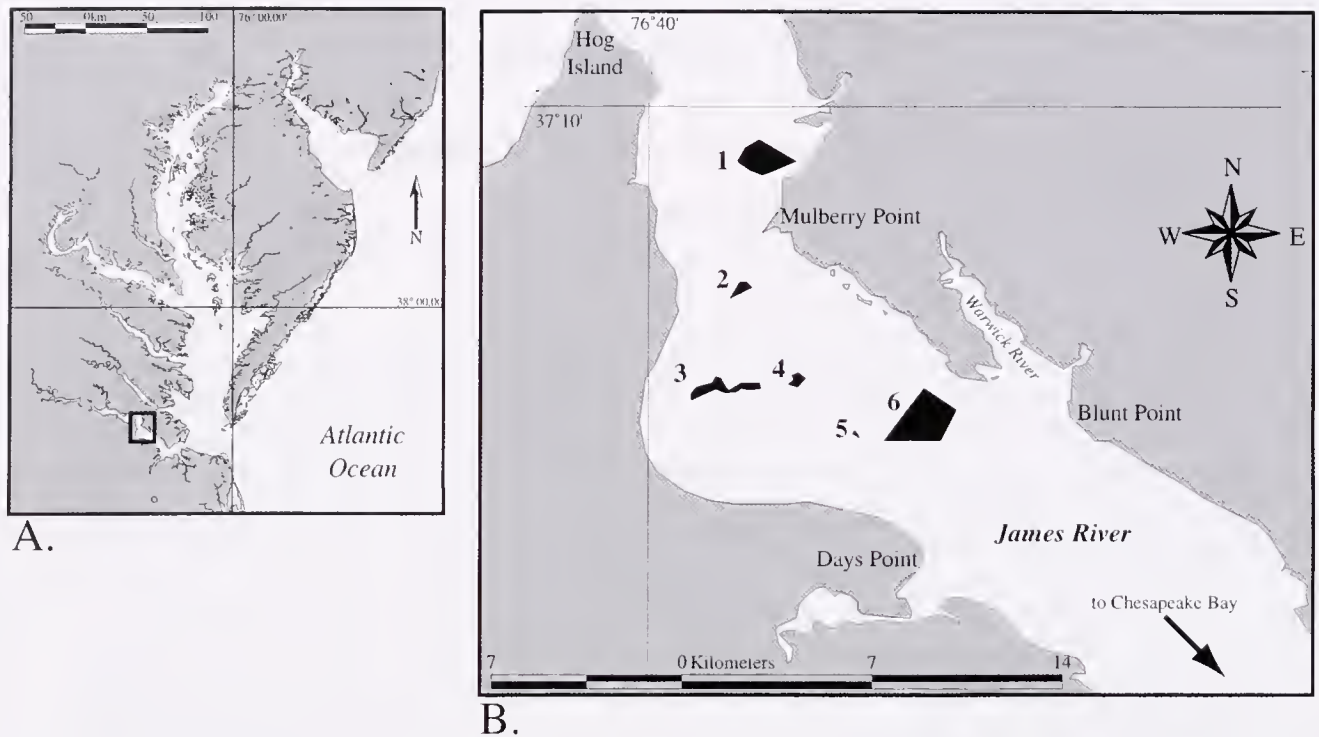


Figure 1. Map of Chesapeake Bay, USA (A) and the James River, Virginia (B) showing the reefs used for sample collection in this study: (1) Deep Water Shoal, (2) Horse Head, (3) Point of Shoals, (4) Cross Rock, (5) Dry Lumps, and (6) Wreck Shoals.

that are probably not well sampled by a dredge, although the sampling insures collection of all oysters within the tong mouth. Since 1998, all oysters collected on the patent tong survey have been measured in the field and length-frequency data have been collected at 5-mm size intervals. Prior to 1998, oysters were simply characterized as small (25–75 mm SL) and market (SL > 75 mm). Total shell volume (liters) is also recorded for each patent tong grab.

In addition to patent tong sampling the exploited James River oyster stocks are subject to regular examination within surveys effected using a traditional oyster dredge (e.g., dredge mouth 1.2 m wide, 0.1 m long teeth, bag volume of 147.87 L or 4.19 bushels). Oysters from either sample type were described as 1 of 2 size classes (small, market) on the basis of length. Oyster abundances from patent tong surveys were converted to numbers of oysters using the conversion factors of 1000 oysters bushel⁻¹ for small oysters (25–75 mm SL), and 500 oysters bushel⁻¹ for oysters of marketable size (SL > 75 mm). This allows an estimation of the number of oysters bushel⁻¹, the unit given by dredge data, and for patent tong data by dividing the converted abundance values for each size class by the total shell volume bushel⁻¹ recorded at that patent tong grab site. The resulting abundance estimates (oysters of a size class bushel⁻¹) were summed within a single patent tong grab and then all of the grabs within 1 site in 1 year were averaged to provide estimates of average numbers of small, market, and total oysters bushel⁻¹ with standard errors of the mean.

Quantification of dredge data is more difficult than patent tong data in that dredges accumulate organisms as they move over the bottom, may not sample with constancy throughout a single dredge haul, and may fill before completion of the haul, thereby providing biased sampling in favor of the “early” portion of the haul. Al-

though Powell et al. (2002) describe bias against shell in oyster dredge samples, these samples were collected during 1 minute tows in which the dredge never filled. The samples described in this study were all collected by tows of longer duration where the dredge filled completely before the end of the tow. For this study we assume no selective retention of live oysters with respect to shell substrate by the dredge due to the method in which the dredge was fished. Selectivity of dredges versus patent tongs with respect to demographics has not been rigorously examined in Virginia waters, especially in shell-poor substrates where tongs dig deeply into buried shell material. Conversely, dredges provide semiquantitative data, have been used with consistency over extended periods (decades), and thus provide data on population trends. The challenge in the current objective of estimating absolute numbers of oysters is to use dredge data in a consistent quantitative approach.

RESULTS AND DISCUSSION

Within the 1993 to 2001 comparison, we have used the same stations for both dredge and patent tong surveys. Pre 1998 data may well have suffered from subjective distinction of the small versus spat categories in the field. Given the annual abundance of spat, this may have led to inconsistent distinctions of these size categories and occasional large discrepancies in the resulting plot of total oysters per bushel as estimated by dredge and patent tong methods (Figs. 2A, 2C, and 2E). Examination of the data collected at 5-mm size intervals for 1998 to 2001 is illustrated in Figures 2B, 2D, and 2F.

Recalling that both figures give points that are mean values, the plotted relationships represent a considerable body of individual

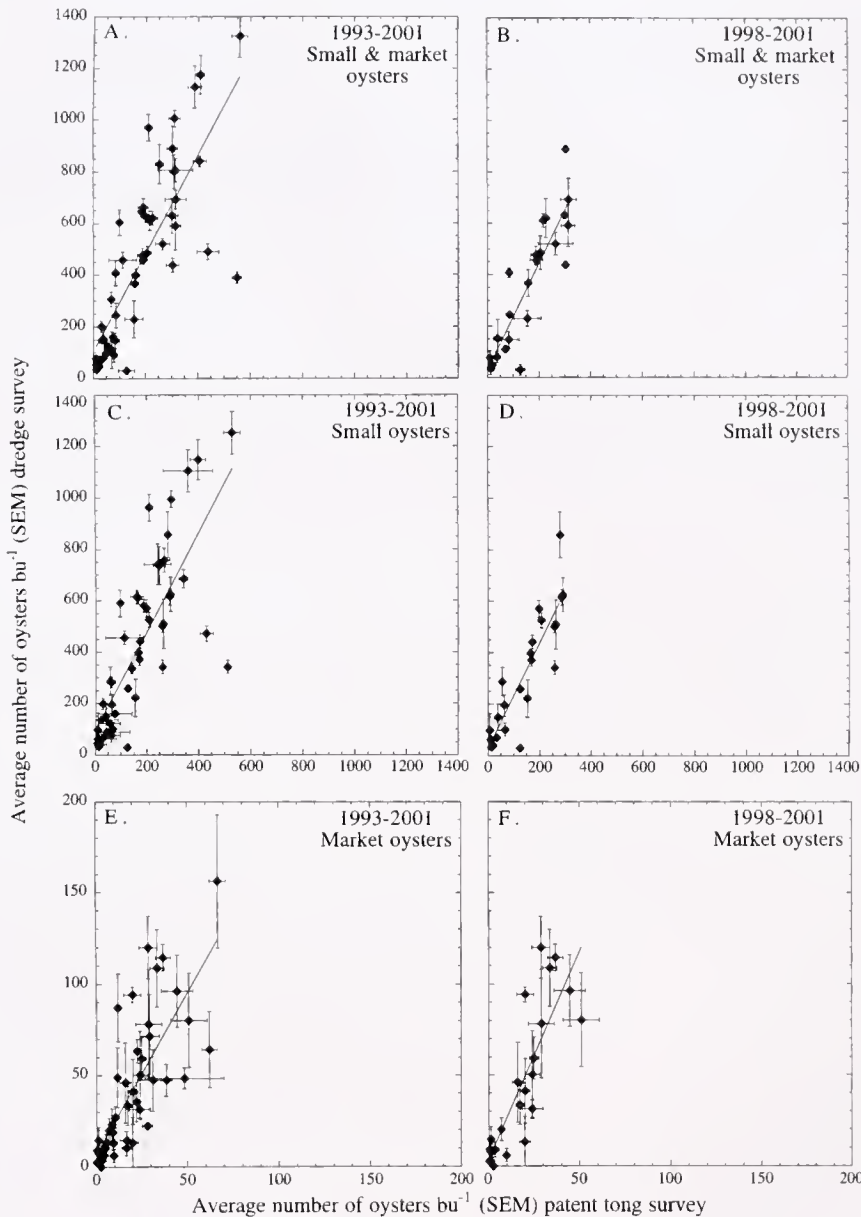


Figure 2. Average total number of oysters (A, B), small oysters (C, D), and market oysters (E, F) bu⁻¹ collected from 6 reefs in the James River with patent tongs in comparison to dredge data. Error bars indicate standard error of the mean. Note the difference in scale between plots A–D and plots E and F. (A) $y = 104.17 + 1.9x$, $R^2 = 0.65$, (B) $y = 27.46 + 2.08x$, $R^2 = 0.79$, (C) $y = 99.31 + 1.9x$, $R^2 = 0.62$, (D) $y = 27.92 + 2.05x$, $R^2 = 0.78$, (E) $y = 6.79 + 1.77x$, $R^2 = 0.62$, and (F) $y = 4.07 + 2.28x$, $R^2 = 0.70$.

data points (annual dredge survey $n > 24$, annual patent tong $n > 130$). All plots illustrate the different results obtained by tong and dredge respectively. These data show that the assumption of no selective retention of live oysters with respect to shell substrate by the dredge due to the method in which the dredge was fished is not correct. The dredge samples appear to be biased towards live oysters with an enrichment of live oysters versus cultch on the order of a factor 2. Similar patterns of dredge sample enrichment have been reported by Powell et al. (2002) for oyster dredges with 1-min tows.

Enrichment in dredge samples may occur because dredging takes the top layer of shell whereas the patent tong also includes a buried layer of shell where it occurs. So more shell per unit oyster

can be expected in a tong sample with a concomitant lower bushel count of oysters—again the historical unit of record. On the other hand, a plot of the grand means of a very large amount of data on a single plot suggests that a line might be fitted to this data. Any attempt to fit a line for predictive purposes through these data would not pass through the origin unless a nonlinear fit was used. The appropriate model for such a fit is open to debate, and may be influenced by the nature of the underlying substrate in many locations—for example oyster reefs in the productive part of the James River have a hard base into which the patent tong does not sink easily. Universal application of a fitted “predicative” relationship may, however, be tenuous. In contrast to the James River, oyster reefs in the Rappahannock River may be less consolidated

resulting in deeper penetration of the underlying substrate. Site-specific application may be more appropriate.

Clearly, annual surveys using standardized methods and sampling designs can provide a wealth of relevant stock assessment information, provided caution is used when extrapolating site-specific data beyond regions that have been surveyed. This exercise demonstrates, different methods and provide slightly different estimates of the available resources, and these estimates are strongly affected by modest changes in available substrate per unit area.

ACKNOWLEDGMENTS

This work was supported by NOAA Chesapeake Bay Stock Assessment Committee (NA66FU0487 and NA17FU2888), the NOAA Office of Sea Grant (NA56RGO141) and EPA Chesapeake Bay Program (CB983649-01-0). This is VIMS Contribution Number 2613 from the School of Marine Science, Virginia Institute of Marine Science. The assistance of survey staff at VIMS and VMRC is gratefully acknowledged.

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DECADAL SCALE CHANGES IN SEASONAL PATTERNS OF OYSTER RECRUITMENT IN THE VIRGINIA SUB ESTUARIES OF THE CHESAPEAKE BAY

MELISSA SOUTHWORTH* AND ROGER MANN

Virginia Institute of Marine Science, College of William and Mary, P.O. Box 1346,
Gloucester Point, Virginia 23062

ABSTRACT Reproductive periodicity of sessile estuarine invertebrates reflects local seasonality of environmental (temperature, salinity) and biologic (food) parameters. Estuaries are ephemeral features in geologic time but considered somewhat constant in the course of recent human history (decadal time scales). Analyses of long-term trends in eastern oyster (*Crassostrea virginica*) settlement periodicity since 1960 in three major Chesapeake Bay rivers (James, Piankatank and Great Wicomico Rivers) of the Chesapeake Bay show marked changes within the 4-decade time frame. The 50th percentile of cumulative recruitment occurs between day 194 and 250 of the year depending on year and location. Significant coherence in interannual variation is observed across a wide range of sites. These patterns are related to pre and post disease (both *Haplosporidium nelsoni* and *Perkinsus marinus*) events, periods characterized by high and low river flow, varying harvest pressure, and trends arguably associated with directed climate change.

KEY WORDS: eastern oyster, *Crassostrea virginica*, settlement, recruitment, climate change, Chesapeake Bay

INTRODUCTION

Sessile marine invertebrates are suitable candidates to examine long-term changes in climate and anthropogenically induced changes in local environments. Recruitment intensity and periodicity are annual signals of the integrated impact of local perturbations superimposed on long-term (geological scale) changes. Commercially valuable species have been the focus of quantitative annual monitoring programs in support of fishery management, but have been examined in a limited manner with respect to combined impacts of climate change and fishing pressure (Allen & Turner 1989, Kim & Powell 1998). Estuarine environments are particularly susceptible to stress from cyclical changes on time scales ranging from tidal to annual. Long-term data on marine invertebrate communities in estuaries are limited, especially so in regions subject to increasing watershed development, water quality degradation, habitat destruction and/or diseases, and parasites. Temperate estuaries are natural laboratories where cumulative impacts of human societal growth are highly visible. Eastern oysters, *Crassostrea virginica*, are considered sentinel organisms in estuaries on the North American Atlantic seaboard in terms of biologic and geologic (habitat) function. Their loss in such environments predicates significant changes in ecosystem function and food chain dynamics with trickle down effects on nutrient cycling, species richness and complexity, stability of food webs, and production to support commercial fisheries.

The eastern oyster has long been recognized for its ecologic and commercial importance in the Chesapeake Bay, but the species has suffered numerous insults over the past century. Over fishing of oysters in the Chesapeake Bay has long been recognized. Maryland's harvests have been in decline since about 1885 and Virginia's since about 1904 (Hargis & Haven 1995). Recent catches are less than 1% of what they were 100 years ago. In addition to the continuous removal of market and seed oysters, uncounted millions of tons of shell have been removed for use in road building, chemical processing, and poultry husbandry. This essential habitat loss has resulted in the gradual replacement of 3-dimensional intertidal reefs, with 2-dimensional, subtidal reefs that are highly susceptible to siltation and burial (Hargis & Haven 1995). The

onset of *Perkinsus marinus* in 1950 (Andrews 1996) and the arrival of the non-native disease *Haplosporidium nelsoni* (MSX) in 1959 (Burrison et al. 2000) caused further decline in the already seriously depleted oyster populations. Despite efforts of replenishment, beginning as early as 1924 in Maryland and 1928 in Virginia, oyster stocks have continuously declined. In response to these accumulating problems, monitoring efforts increased and became routine starting in the late 1940s and early 1950s in both Maryland and Virginia (Andrews 1982).

Early studies on oyster settlement (spat or young of the year oysters undergoing metamorphosis and attaching to the bottom) and recruitment (those oysters that survive post settlement to become part of the population) in Virginia focused on seasonal patterns in onset, duration, intensity, and cessation of oyster settlement (Andrews 1951, Andrews 1954). With the onset of the diseases, *P. marinus* and *H. nelsoni*, in the late 1950s and 1960s these patterns changed. Population studies from 1946 to 1967 in the James River showed that post *H. nelsoni* settlement was of lower intensity and occurred during a shorter period when compared with pre *H. nelsoni* settlement (Andrews 1982).

There have been two other long-term studies of oyster settlement and recruitment in the Virginia portion of the Chesapeake Bay. Haven and Fritz (1985) focused on the temporal and spatial distribution of oyster settlement. They examined weekly settlement in the James River from 1963 to 1980. They separated the river into three distinct settlement zones related to water circulation and found that settlement was synchronous at stations within a zone, but occurred 1 to 2 weeks earlier at stations in the upriver zones compared with the downriver zones. They also found that post *H. nelsoni* settlement intensity was lower and occurred in discrete pulses, 1 to 2 weeks in duration, instead of the continuous settlement pattern seen in pre *H. nelsoni* conditions. Austin et al. (1996) performed a time series analysis of recruitment from 1946 to 1993 in the four major sub estuaries of the Virginia portion of the Chesapeake Bay. The data used were from the Virginia Institute of Marine Science's (VIMS) annual fall dredge survey. They found a relationship between spat (young of the year, recently settled oysters) and subsequent seed at 2- and 3-years post settlement, but no relationship between recruitment numbers and spring and summer water temperatures and river discharge. Their study provided an overall picture of interannual variation in recruitment.

*Corresponding author. E-mail: melsouth@vims.edu

but did not provide any information on interannual variation in the onset and duration of oyster settlement. Whereas both of these studies provide insight into oyster settlement and recruitment in the larger rivers of the Virginia portion of the Bay, relatively little effort has been devoted to critical examination of changes in settlement patterns in some of the smaller rivers. VIMS provides a descriptive monitoring report of settlement and recruitment in both small and large rivers in Virginia (summaries available at <http://www.vims.edu/mollusc>) on an annual basis. Oyster settlement in these smaller systems and the overall health of resident oyster populations has become increasingly important over the past decade, primarily due to increasing restoration efforts in these smaller systems. The Piankatank and Great Wicomico Rivers in particular have served as important building blocks in a long-term plan for oyster restoration in Virginia (Bartol & Mann 1997, Southworth & Mann 1998).

In this study, we examine long-term changes in the Chesapeake Bay oyster population in response to the cumulative effects of the previously described stressors. We report the long-term trends in periodicity of oyster settlement in 3 rivers, namely the James, Piankatank, and Great Wicomico. These systems offer contrasting watershed drainage areas, river flows, and basin morphologies. We use duration of settlement period and mean date of settlement as indicators of environmental quality within a single year for each location. Comparisons were made within and between river systems over a 40-year period, to examine the relative roles of large-

scale climatic events, local physical functions, and biologic stressors in driving the settlement patterns.

The value of historical long-term data sets, such as the VIMS shellstring survey used in this study, is in the consistency and length of data collection. Due to this longevity, the data can provide valuable insight into long-term trends (on a decadal or longer time scale) that most experiments do not afford. However, given that the initial data collection often has a different objective (i.e., was not designed to examine long-term trends) we have adopted caution in the analysis and interpretation of the data. As such we provide an overview of oyster settlement timing based on the long-term data set in a largely descriptive manner, using statistics that are appropriate to the data set.

MATERIALS AND METHODS

The James River (Fig. 1) has a large watershed, (approximately 27,000 km², Chesapeake Bay Program; <http://www.chesapeakebay.net>) with yearly average stream flow ranging from 4,400 to 21,500 ft³/sec (United States Geological Survey data; <http://nwis.waterdata.usgs.gov>). Historically it was a major seed-producing river, with the seed area extending approximately from the Nansemond River up to Deep Water Shoal (Fig. 1), that supplied Virginia and Maryland planters with an average of 2 million bushels each year (Andrews 1982). The Piankatank and Great Wicomico Rivers (see Fig. 1) are relatively small watersheds (approximately 575 and 337

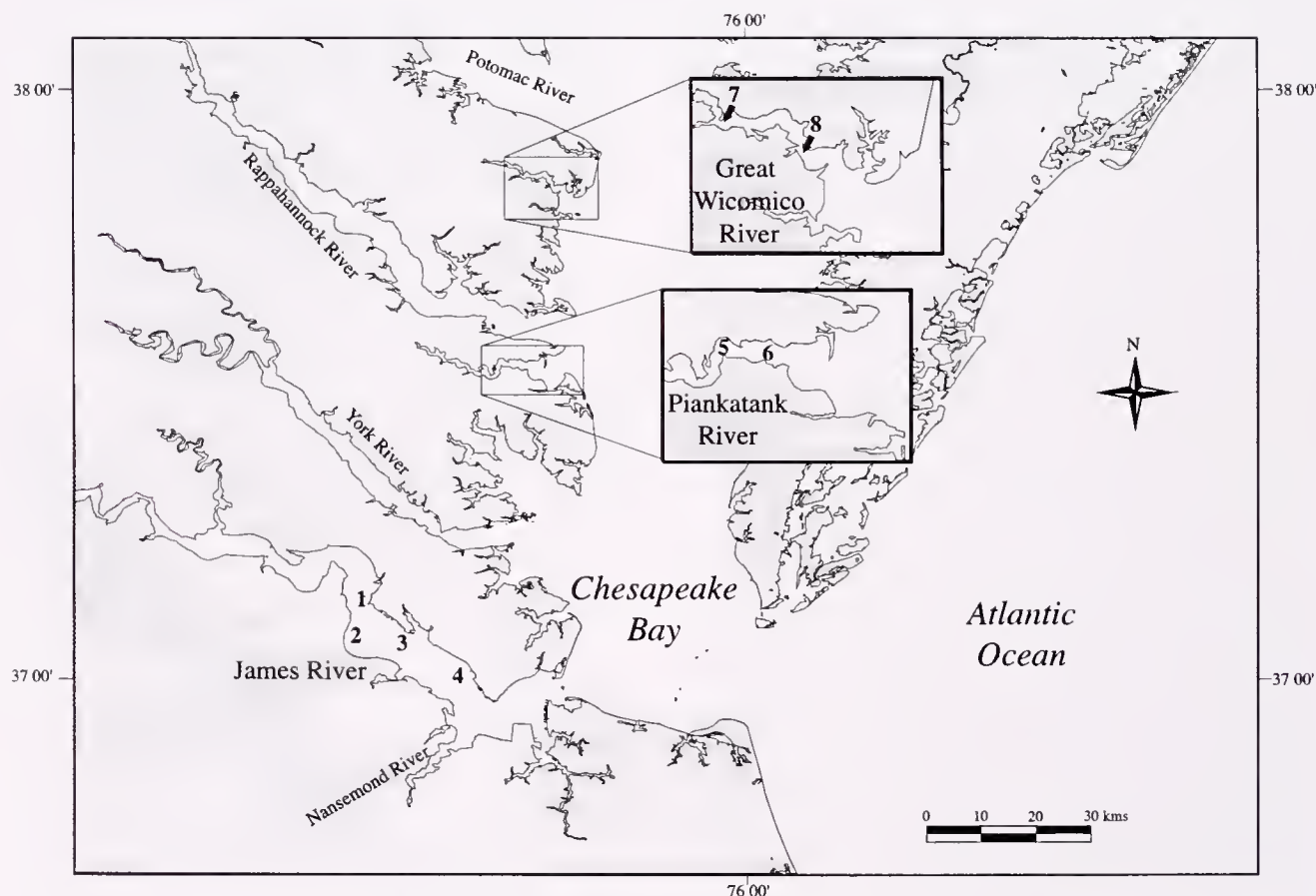


Figure 1. Map of the Chesapeake Bay showing the locations of the James, Piankatank, and Great Wicomico Rivers and the location of the 8 study sites (numbering system used throughout figures): (1) Deep Water Shoal, (2) Point of Shoal, (3) Wreck Shoal, (4) Miles Watch House, (5) Ginney Point, (6) Palace Bar, (7) Glebe Point, (8) Hudnall.

km², respectively, Chesapeake Bay Program; www.chesapeakebay.net) with a yearly average stream flow ranging from 50 to 460 ft³/sec in the Piankatank River and 57 to 266 ft³/sec in the Great Wicomico River (U.S. Geological Survey data; <http://nwis.waterdata.usgs.gov>). Both rivers have been termed trap-type estuaries by Andrews (1979) and are characterized by gyre-like circulation in their lower reaches. Historically, these rivers were used for oyster seed production and grow-out to market size on leased grounds. More recently, these rivers have become important in restoration efforts, with both rivers receiving shell plants and artificial oyster reefs (Southworth et al. 2002, Berman et al. 2002).

VIMS monitors oyster settlement annually using shellstring substrates from late May to early June through October. A shellstring consists of 12 oyster shells of similar size (standard length 76 mm.) drilled through the center and strung (inside of shell facing substrate) on heavy gauge wire (Fig. 2). Throughout the monitoring period, shellstrings were deployed approximately 0.5 m off the bottom at each station. Shellstrings were usually replaced after a 1-week exposure. The number of spat that attach to the smooth underside of the middle 10 shells during the exposure period were counted under a dissecting microscope. An estimate of the mean number of spat shell⁻¹ for the exposure period



Figure 2. Picture of a typical shellstring used by the Virginia Institute of Marine Science Molluscan Ecology Program to monitor oyster spat settlement in Virginia estuaries, 1946 to present.

TABLE 1.

Summary of the Virginia Institute of Marine Science oyster settlement monitoring stations in the James, Piankatank, and Great Wicomico Rivers, Virginia.

Station # (from Fig. 1)	River System	Station	Years Covered by Survey	Total Number of Years
1	James	Deep Water Shoals	64-72, 74-02	38
2	James	Point of Shoals	68-02	35
3	James	Wreck Shoal	68-02	35
4	James	Miles Watch House	63-97	35
5	Piankatank	Ginney Point	65-02	38
6	Piankatank	Palace Bar	67-02	36
7	Great Wicomico	Glebe Point	65-02	38
8	Great Wicomico	Hudnall	65-02	38

was obtained by dividing the total number of spat observed by the number of shells examined (10 shells in most cases). This estimate was then standardized on a per week basis (day 1 of the year = January 1) to allow for comparison between years and calculation of cumulative spat shell⁻¹ estimates for each year.

A total of 8 sites were selected for the study, 4 in the James River and 2 each in both the Piankatank and Great Wicomico Rivers with 34 to 38 years of data per site (Table 1). Figure 1 shows the location of the sites in their respective rivers. Sites were chosen based on their location in the river and consistency of collection of data. Data analyses were as follows. A sigmoid curve was produced for each year at each site using the formula:

$$Y = \frac{a}{1 + e^{\frac{-(x-x_0)}{b}}}$$

Where:

a = the maximum y (i.e., the total number of spat shell⁻¹ in a given year).

b = the maximum slope of the line.

X_0 = 50th percentile of cumulative recruitment (i.e., the day of the year when 50% of " a " has been obtained).

Examinations of temporal trends in X_0 were made by plotting site-specific values of X_0 for the multiyear duration of the data set. A common characteristic of time series data sets is the illustration of different features and patterns over different time scales. For example, there may be large interannual variation in a particular data set, but the overall trend on a larger decadal scale may show a steady decline. Smoothing is a tool available to identify trends within long-term data sets. One such smoothing technique, developed by Cleveland (1979), is Loess (originally LOWESS or LOcally WEighted Scatterplot Smoothing), which applies the tricube weight function to weight the data. A weighted regression is performed for each point along the smooth curve. Loess obtains each point along the smooth curve by performing a regression on the data points close to the curve point where the closest points are more heavily weighted. The user determines the amount of smoothing, which affects the number of points in the regression. This technique is robust and sufficiently well accepted to be included in most statistical packages (Cleveland 1979, 1993).

Temperature is generally considered to be an important ecological parameter influencing reproductive periodicity and larval development rates in oyster populations (Thompson et al. 1996).

We sought to examine the influence of temperature on periodicity and intensity of oyster settlement in the long-term data sets. Whereas temperature data goes back to the early 1980s for the James River and the early 1990s for the Piankatank River, consistent data for all three systems was only available from 1998 through 2002. Therefore, we limited comparisons and analysis to those 5 years. Temperature residuals were calculated by subtracting the temperature in the James River from that in the Piankatank and Great Wicomico Rivers for each day throughout the spawning season. A positive result can be interpreted as that system being warmer than the James River, whereas a negative result means the system is colder than the James River. The sum of the residuals for a particular river throughout the spawning season describes the magnitude of the difference between the river of interest and the James River.

Records of salinity over the time period examined in the three systems are also very limited; however, there are comprehensive river flow data near Richmond in the James River and near Dragon Swamp in the Piankatank River for the entire time period and in Bush Mill Creek (which flows into the Great Wicomico River) from 1963 through 1986, from the United States Geological Survey (<http://nwis.waterdata.usgs.gov>) records. Whereas river flow data does not give us a direct measure of salinity, it can be used as a measure of relative salinity in that there is an inverse relationship between river flow and salinity (Mann & Evans 1998).

RESULTS AND DISCUSSION

The relationship between day of the year and the cumulative sum of spat shell⁻¹ was accurately described using the fitted sigmoid curve (R^2 values >0.92) and an example curve from each river is shown in Figure 3. There was a wide range in X_0 over the period examined in all three rivers. Over the 40-year period there was as much as a 60 to 90 day difference in the timing of oyster settlement between years and between river systems (Fig. 4). This plot demonstrates variability in oyster settlement timing between systems and years, but fails to identify any trends that may exist in the data. Therefore the data was smoothed using Loess and the resulting curves are shown in Figure 5.

The smoothed curves still illustrate the large range in the timing of oyster settlement observed over the 40-year period. Aside from the early years (through 1970) in the James River, settlement timing between sites within the same river was similar (usually within 1 week of each other). The Great Wicomico River shows the largest variation in timing, whereas the Piankatank River was fairly consistent in terms of settlement timing, especially compared with the other two systems. Prior to the mid 1970s, settlement was consistently earlier in the Great Wicomico and Piankatank Rivers than in the James River and there was a large difference in settlement timing between the systems. Settlement in the James River tended to be late in the year with the majority of oyster settlement occurring from late August into early September. There was a trend toward earlier settlement in both the James and Great Wicomico Rivers throughout the late 1970s and 1980s. Beginning in the early 1990s settlement timing in all three systems was increasingly later in the year and has remained similar (within 3 to 4 weeks of each other) since then. In particular, oyster settlement in the James River appears to have undergone the largest change such that current settlement patterns are similar to the other two systems; however, settlement in the Great Wicomico remains about 2 weeks earlier than in the other two systems.

There are many environmental factors that have the potential to affect both the timing and magnitude of oyster settlement. Among these are single large-scale meteorologic events, which may temporarily but fundamentally alter the conditions in a system, such as tropical storms or hurricanes (Haven et al. 1974), temperature (Medcof 1939), salinity (Butler 1949), disease (Ford & Figueras 1988, Choi et al. 1989), and location of broodstock in a system (Haven & Fritz 1985). One or all of these factors may explain the variability in oyster settlement timing observed over the past 35 to 40 years in these rivers.

Throughout the duration of the study, the most significant meteorologic event to occur during a settlement season was Hurricane Agnes that entered the Chesapeake Bay in June of 1972 and resulted in record amounts of flooding in most of the major sub estuaries (Andersen et al. 1973, Schubel et al. 1974). This flooding had a major effect on oyster populations in the Bay causing 2 to 70% mortality in adult oysters (Haven et al. 1974). The mortality was mostly limited to the shallower systems and the upper bay and upriver sites. Mortality in the James River was as high as 85% at the more upriver sites, but was relatively low and similar to normal years at the more downriver sites, where the majority of the broodstock was located (Haven et al. 1974). Hurricane Agnes was responsible for almost complete recruitment failure in 1972 and severely reduced settlement in 1973 (Haven et al. 1974). Despite these short-term effects, Hurricane Agnes seems to have had little effect on the long-term trends in timing of the set in the Virginia portion of the Chesapeake Bay (Fig. 5).

Temperature and salinity affect every aspect of an oyster's biology, including gonadal development and timing of the spawn. Temperature in particular is viewed as the single most important factor controlling when the eastern oyster spawns (Shumway 1996). Figure 6 shows the daily temperature residuals for the 1998 to 2002 period for the Piankatank and Great Wicomico Rivers compared with the James River. In general, the temperature in the Great Wicomico River tends to be 1 to 2 °C warmer than the James River, whereas the temperature in the Piankatank River tends to be about 1 °C cooler than the James River. Both smaller rivers exhibit similar early season increases of 2 to 4 °C over a short (2 to 3 weeks) time period when compared with the James River. Further exploration of this temperature relationship can be examined by observing the cumulative day degrees as shown in Figure 7. The cumulative day degree is a sum of all of the temperature residuals for a particular year and system and demonstrates that the Great Wicomico River is, on average, warmer than the James River whereas the Piankatank River is, on average, cooler than the James River.

The difference in temperature between the three systems may explain several aspects of the observed settlement trends (see Fig. 5). Throughout the observed time span, spawning in the Great Wicomico tended to occur 1 to 2 weeks earlier than in the other two systems. Whereas both the Piankatank and the Great Wicomico Rivers showed a pronounced increase in temperature early in the season when compared with the James River (see Fig. 6), the Great Wicomico River also was on average several degrees warmer than the other two systems. Several studies have found that the rate of temperature change can be as important in inducing spawning in oysters as some "critical" level being obtained (Medcof 1939, Butler 1949). If we assume the temperature residuals in these systems have remained relatively consistent over the past 40 years then the increase in temperature observed in the Great Wicomico River early in the season combined with the overall

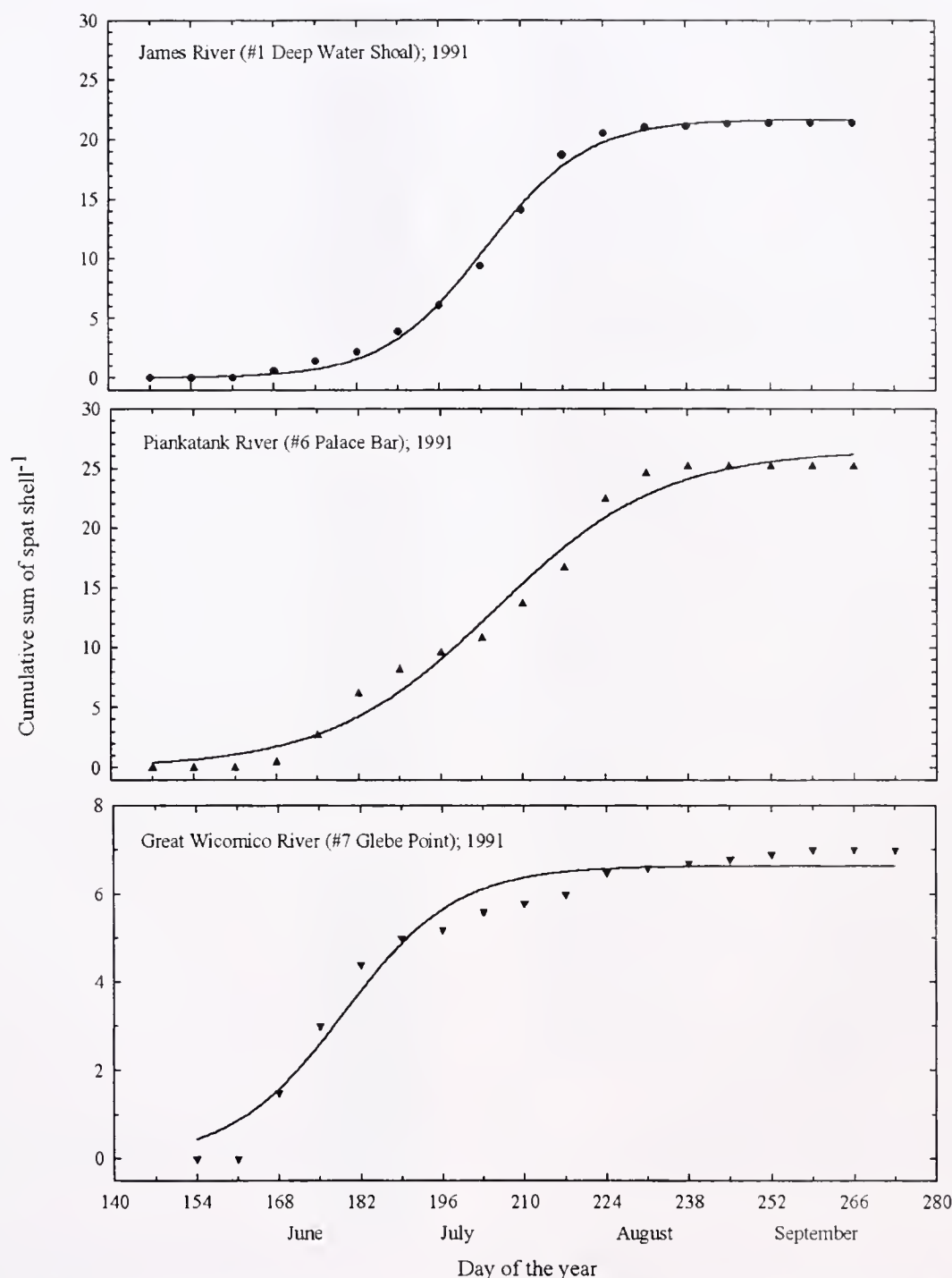


Figure 3. Examples (from 1991) of the sigmoidal curve fit of day of the year versus cumulative sum of spat shell⁻¹ from Deep Water Shoal (#1) in the James River (top), Palace Bar (#6) in the Piankatank River (middle) and Glebe Point (#7) in the Great Wicomico River (bottom). Note the difference in y-axis scales between panels.

higher temperatures obtained throughout the spawning season may explain the earlier settlement observed in that system. The Piankatank River seems to warm at a similar rate to the Great Wicomico early in the spawning season, but may take longer to reach that "critical" temperature necessary to induce spawning.

Salinity, while not as important as temperature in determining the timing of spawning, can still affect gametogenesis, especially in flood conditions. Butler (1949) found that gametogenesis was

delayed in salinities less than 6 ppt. Laboratory examination of gonads from field collected animals from May to August showed a 2-month lag in gametogenic development in about 90% of the oysters from a low salinity site (0–6 ppt) when compared with a high salinity site (6–15 ppt; Butler 1949). Whereas a few oysters did undergo normal gametogenesis and spawn at low salinity, the majority of them failed to produce gametes until salinity rose above 8 ppt (Butler 1949).

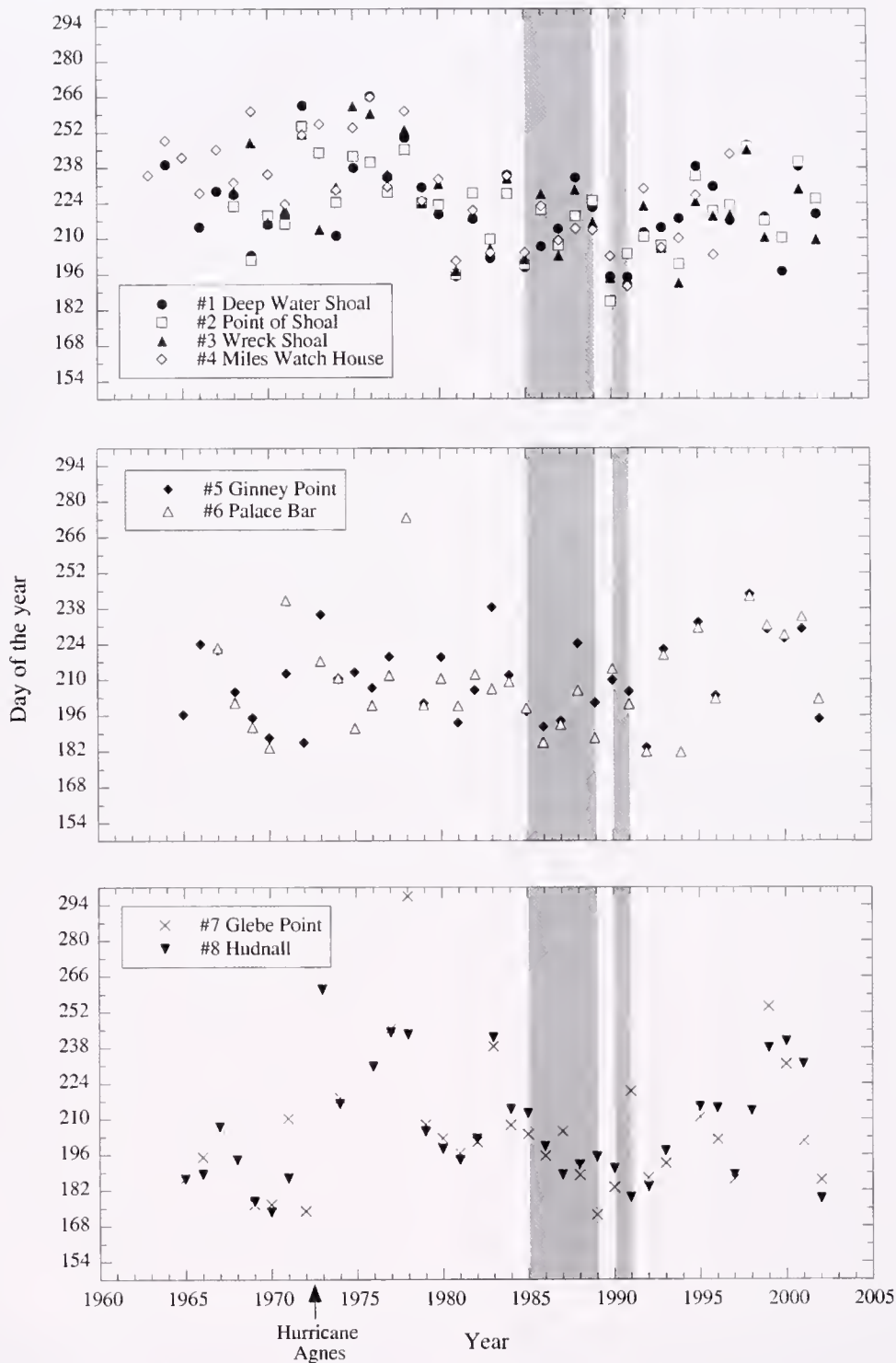


Figure 4. Plot of X_{50} (day of the year when 50% of the total settlement has occurred) over the 40-y study for the James (top), Piankatank (middle) and Great Wicomico (bottom) Rivers. Station numbers correspond with Figure 1 and Table 1. Shaded regions represent dry years as discussed in text.

River flow has been shown to have an inverse relationship with salinity (Mann & Evans 1998). Figures 8, 9, and 10 show average monthly water flow residuals (difference from the long-term average) from USGS records for May through September from 1960 through 2002 in the James and Piankatank Rivers and from 1964 through 1986 in the Great Wicomico River respectively. The water

stations where data was obtained for each system drain approximately 65%, 45%, and 5% of the total watershed of the James, Piankatank and Great Wicomico Rivers, respectively. Applying corrections to the reported raw data for watershed area and the percentage of watershed reflected in the raw data we note that the run-off in the Piankatank and Great Wicomico Rivers are in the

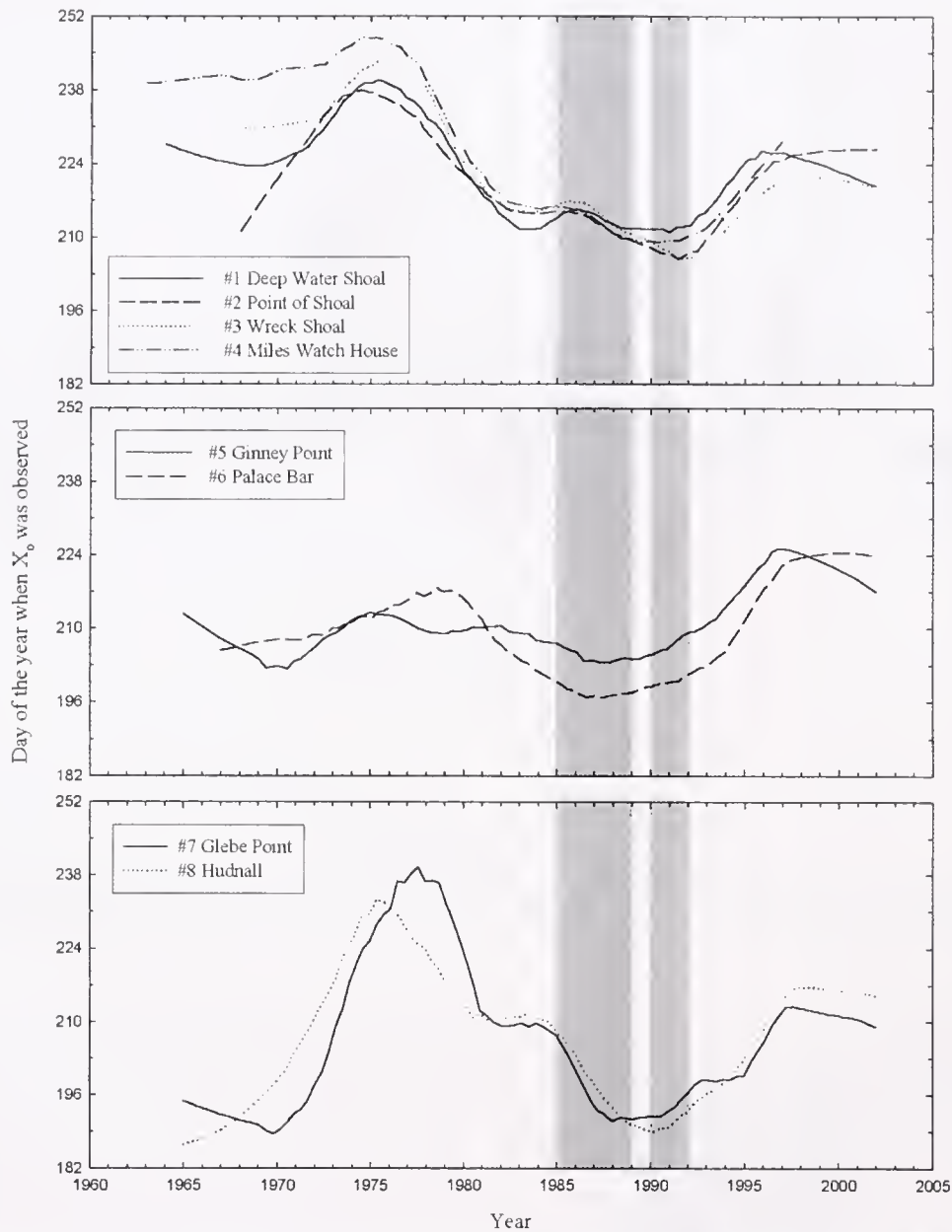


Figure 5. Cumulative recruitment (X_0) data for the James (top), Piankatank (middle) and Great Wicomico (bottom) Rivers, smoothed with the Loess technique in relation to year. Station numbers correspond with Figure 1 and Table 1. Shaded regions represent dry years as discussed in text.

10s to 100s of cubic feet sec^{-1} whereas the James is in the 1,000s to 10,000s cubic ft sec^{-1} , at least two orders of magnitude higher. All three systems were characterized by low flow during the 1960s. Hurricane Agnes (June, 1972) appeared to have disproportionate impact in the James River compared with the other two systems. During the 7-year period from 1985 through 1991, only 1989 was considered a wet year for the Chesapeake Bay as a whole (Burrenson & Ragone Calvo 1996). The 1985 to 1991 drought was more apparent in the James River where 5 of the 7 years had a net flow lower than average whereas only 3 out of the 7 years showed a net flow lower than average in the Piankatank River. Further examination of the relationship between oyster settlement and year (see Fig. 5) for the drought conditions of the late 1980s and early

1990s shows that the oysters spawned earlier in the year than during wetter years.

Salinity can have an indirect effect on oyster spawning through its influence on oyster diseases. Two oyster pathogens, *Haplosporidium nelsoni* and *Perkinsus marinus* were present in varying intensities in all three systems throughout the 40-year time frame of the study. Both diseases have been shown to have a detrimental effect on development of the gonad, especially in heavily infected animals (Choi et al. 1989, Ford & Figueras 1988). Therefore we examined the option that the heavier the infection, the greater effect that infection would have on the animals. The distribution and abundance of both diseases is primarily controlled by salinity (Burrenson & Andrews 1988). *H. nelsoni* requires a salinity of

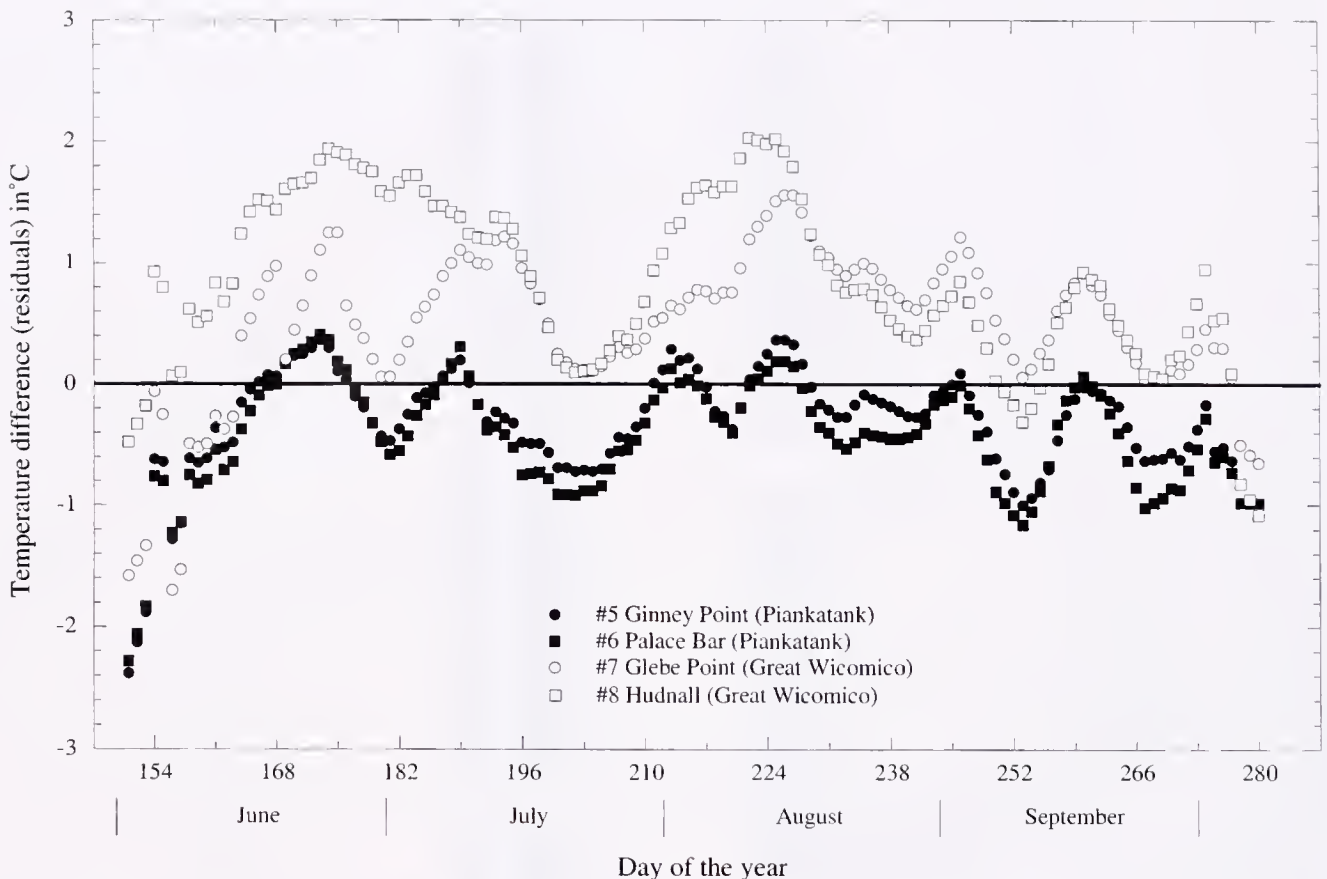


Figure 6. Daily average temperature differences (residuals) in the Piankatank and Great Wicomico Rivers as compared with the James River over the course of the annual spawning season; averaged from 1998 to 2002 when detailed water data was available for all 3 systems. Station numbers correspond with Figure 1 and Table 1.

approximately 15 ppt to infect oysters and is expelled in spring if salinities remain less than 10 ppt for more than 10 days (Andrews 1988). *P. marinus* requires salinities of approximately 12 ppt and can persist for several years at low seasonal salinities without causing substantial mortalities (Burrenson & Andrews 1988). Thus *H. nelsoni* only moves into the upper bay and the upper reaches of the sub estuaries during drought conditions. *P. marinus* showed similar distributions to *H. nelsoni* until drought conditions persisted for the 7-year period in the late 1980s and early 1990s (see Figs 1 and 2 in: Burrenson & Ragone Calvo 1996). Since that time *P. marinus* has persisted in the upper James River and throughout the Piankatank and Great Wicomico Rivers even though salinities returned to normal (compared with the long-term means) during the mid to late 1990s.

The effect that the two diseases have on oyster spawning, especially that of *P. marinus*, may explain the observed changes in settlement timing in the James River during the late 1980s. As disease became more prevalent throughout the James River, the difference in settlement timing between the most upriver (Deep Water Shoals) and downriver (Miles Watch House) stations decreased (see Fig. 5). The observed differences between the 3 rivers have also decreased as disease prevalence has increased.

The change in settlement timing in the James River may be related to the location of the broodstock oysters in that system and how that location has changed over the study period. It has been suggested that, historically, the majority of the settlement on the

upper seed river area (upriver of Wreck Shoal, see Fig. 1) originated from the oysters located in the lower, more saline, part of the river (Haven & Fritz 1985). With the onset of *H. nelsoni* in 1959 many of the oysters in the lower part of the river were killed. Further excursions of both diseases into the upper reaches of the James, throughout the seed area and especially that observed over the past fifteen years, has led to further decline of the downriver broodstock populations. Data from the annual VIMS fall dredge survey (<http://www.vims.edu/mollusc>) show that the percentage of broodstock upriver of Wreck Shoal (Fig. 1 for location in river) has been steadily increasing, whereas the broodstock downriver of Wreck Shoal has been decreasing (Fig. 11). We suggest that, historically, the oysters in the upper seed area provided the first smaller settlement pulse, whereas the more downriver oysters provided the larvae for the major settlement events that typically occurred in late August and early September. With the decline of these downriver populations, the majority of the settlement event increasingly originates from the upper seed area, with an accompanying earlier settlement peak.

In summary, the trends in oyster settlement timing observed over the past 40 years in the James River can be attributed to several interacting factors. There are anthropogenic inputs in the form of watershed influences and over harvesting that have been occurring in the river for the past century (Hargis & Haven 1995) and, despite continuing depleting stocks, some harvesting still occurs in the system (James Wesson, Virginia Marine Resources

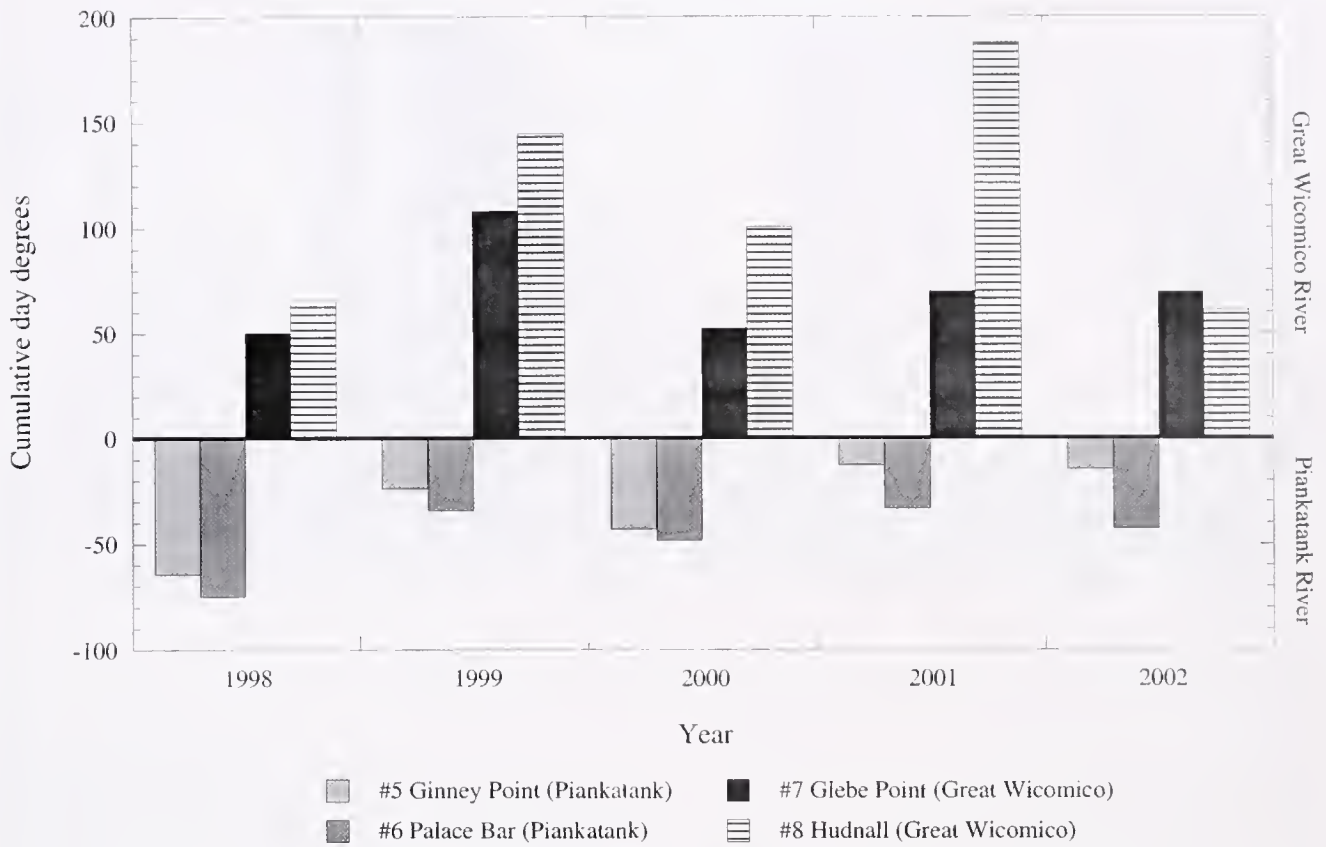


Figure 7. Cumulative day degrees for the Piankatank and Great Wicomico Rivers using the James River as the zero mark for the period 1998 to 2002 when detailed water data was available for all 3 systems. Station numbers correspond with Figure 1 and Table 1.

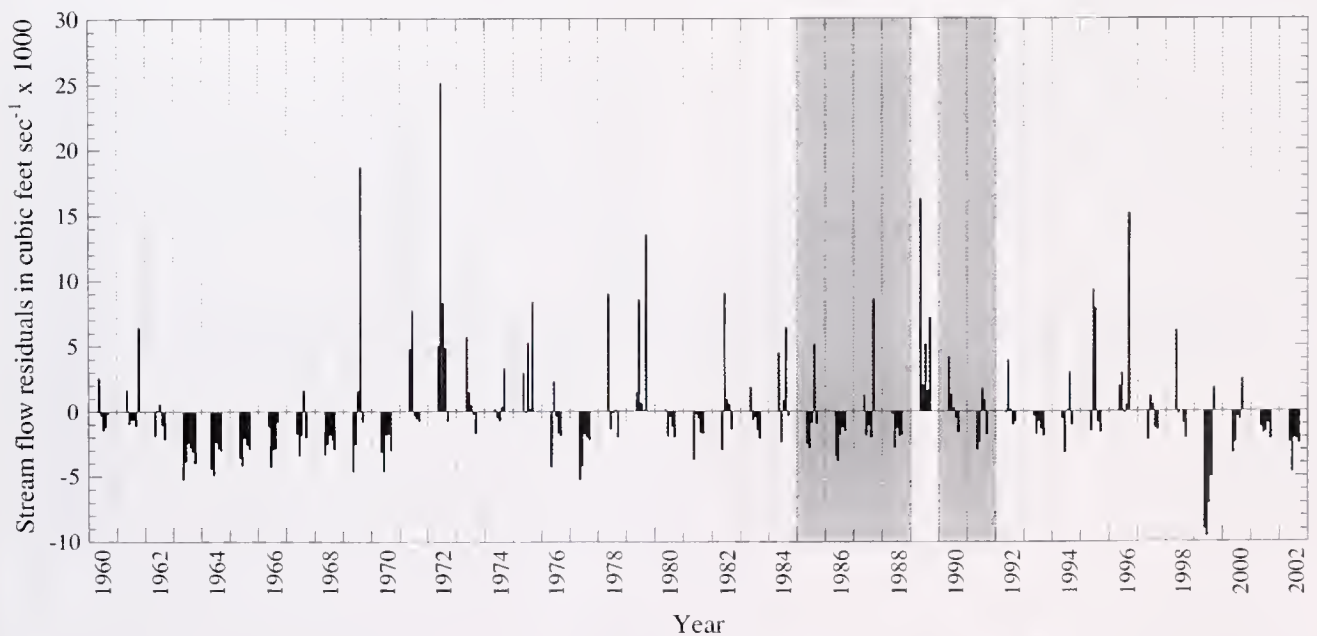


Figure 8. Average monthly stream flow residuals (monthly average minus long-term monthly average) from May through September from USGS records for the James River from 1960 to 2002. Shaded regions represent dry years as discussed in text.

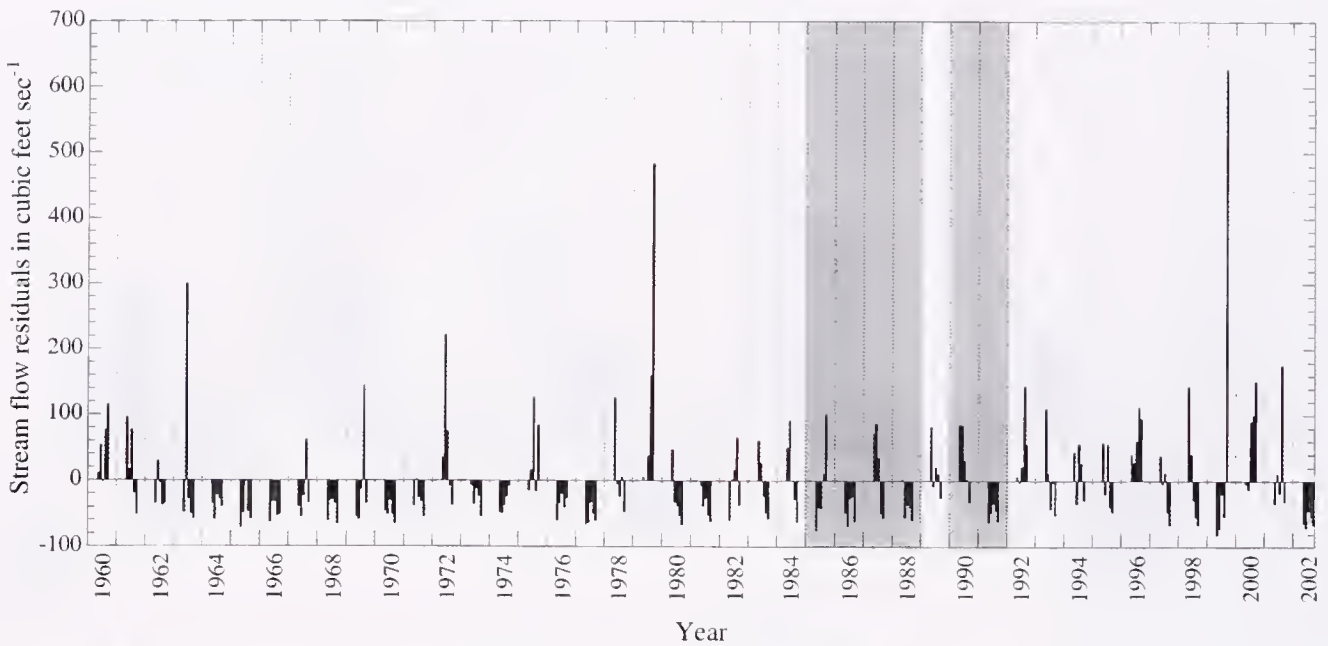


Figure 9. Average monthly stream flow residuals (monthly average minus long-term average) from May through September from USGS records for the Piankatank River from 1960 to 2002. Shaded regions represent dry years as discussed in text.

Commission, Newport News, VA 23607; personal communication). With the added insult of the 2 disease species, *H. nelsoni* and *P. marinus* and the subsequent change in location of the brood-stock populations, the present distribution of oysters in the James River is very different from what was observed several decades ago. Overall there has been very little change in the timing of oyster settlement in the Piankatank River, especially compared with the changes observed in the James and Great Wicomico Rivers. Unlike the James River, there are very few anthropogenic influences in the Piankatank River, there has been no commercial

harvesting in the system for decades (James Wesson; personal communication) and there are few watershed influences. The small change in settlement timing that we observed in the late 1980s and early 1990s are most likely associated with the drought of the late 1980s when *P. marinus* infections moved into the upper portion of the estuary (Burreson & Ragone Calvo 1996). Throughout the duration of the study, the Great Wicomico has exhibited the largest interannual variation in settlement timing. The drought of the late 1980s did not really alter the location of disease-infected oysters (Burreson & Ragone Calvo 1996), and given the small size and

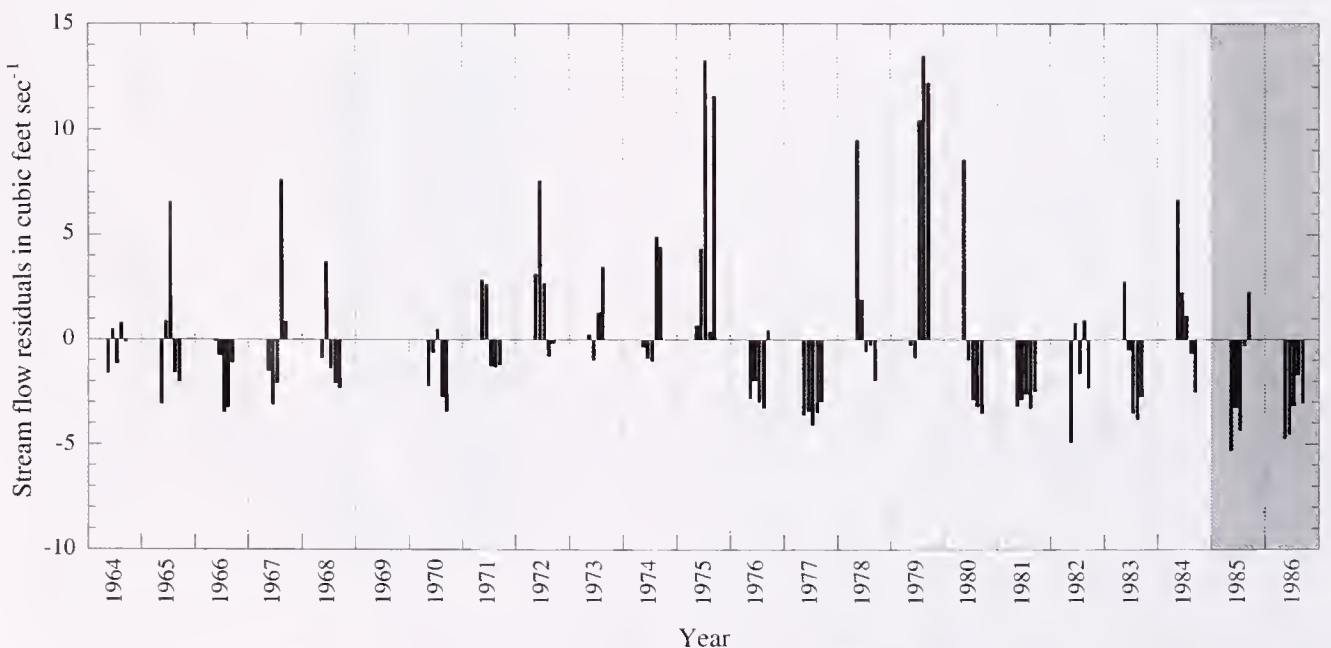


Figure 10. Average monthly stream flow residuals (monthly average minus long-term average) from May through September from USGS records for the Great Wicomico River from 1964 to 1986. Shaded regions represent dry years as discussed in text.

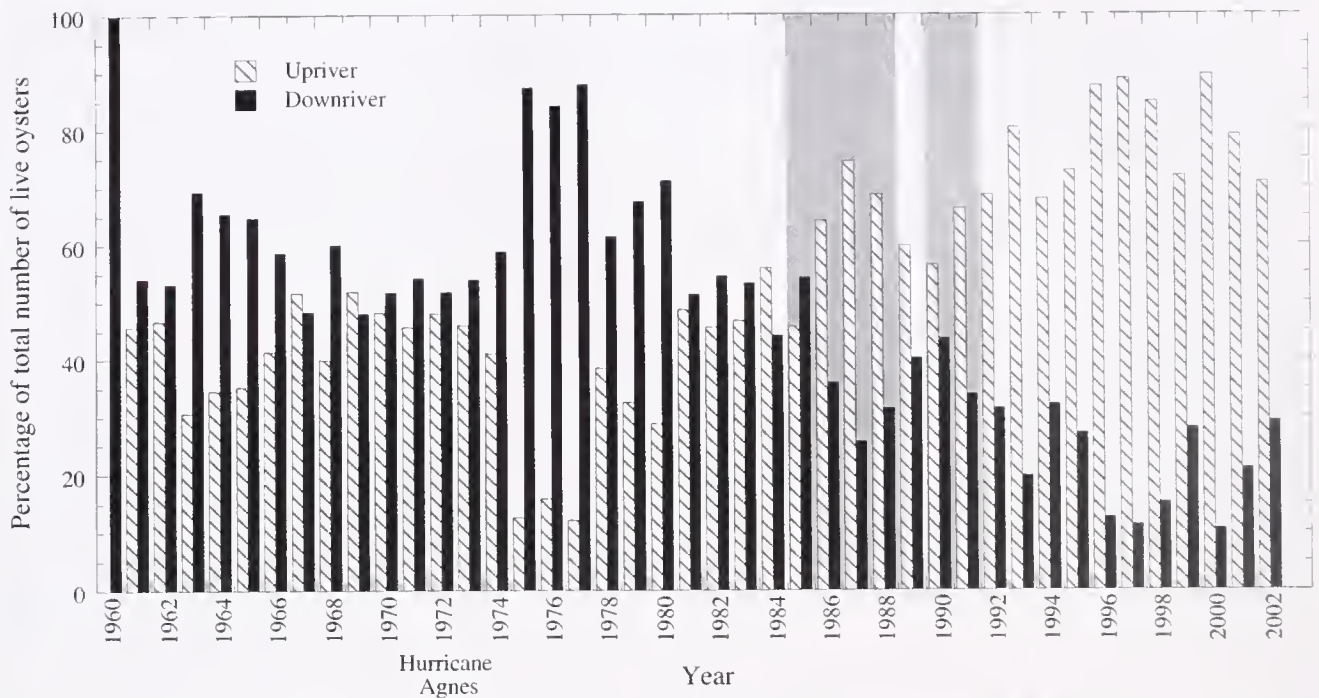


Figure 11. Broodstock location in the James River from the VIMS annual dredge survey from 1960 to 2002: sites upriver of Wreck Shoal (#3) versus sites downriver of Wreck Shoal. See Figure 1 for location within the James River. Shaded regions represent dry years as discussed in text.

gyre-like nature of the sub estuary, location of broodstock has no effect on the timing of oyster settlement. The combination of low run-off and higher temperatures (compared with the other two systems) is implicated and is arguably an effect of directed climate change.

ACKNOWLEDGMENTS

This work was supported by NOAA Chesapeake Bay Stock Assessment Committee grant numbers NA66FU0487 and

NA17FU2888, and the NOAA Office of Sea Grant under grant number NA56RGO141, and EPA Chesapeake Bay Program (CB98364901-0). This is Contribution Number 2601 from the School of Marine Science, Virginia Institute of Marine Science. The assistance and discussions of our colleagues Juliana M. Harding and David Evans are gratefully acknowledged. This manuscript is dedicated to the memory of Buzzy Southworth in appreciation of her continual encouragement and support for my career in marine science.

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GAMETOGENIC PROCESSES IN THE PEARL OYSTER, *PTERIA PENGUIN* (RÖDING, 1798) (BIVALVIA, MOLLUSCA)

U. ARJARASIRIKOON,¹ M. KRUATRACHUE,^{1*} P. SRETARUGSA,² Y. CHITRAMVONG,¹
S. JANTATAEME¹ AND E. S. UPATHAM³

¹Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand;

²Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand; ³Burapha University, Chonburi 20131, Thailand

ABSTRACT The aim of the study is to reveal the gametogenic cycle in the pearl oyster, *Pteria penguin*. The specimens were collected from Si-chang Island (Thailand) from January 2000 to January 2001. Gametogenesis was observed in histological preparations of gonads from 142 adults sampled monthly. Spermatogenesis was classified into 10 stages: spermatogonia, 5 stages of primary spermatocyte, secondary spermatocyte, 2 stages of spermatid, and spermatozoa. Oogenesis was comprised of 6 stages: oogonia and 5 stages of oocyte. Three stages of gonad development were identified in the male: developing, mature, and partial spawning. In the female, there were 4 stages of gonad development: developing, mature, partial spawning, and spent. The spawning cycle of the male was observed throughout the year with a peak of partial spawning in March to June (60%) and in December to January (40%). Females spawned in June and July (50%). The water temperature and the salinity were not correlated to the reproductive cycle of *P. penguin*. The results of this study can be applied to the induction of spawning and the production of seed in the aquaculture system of this pearl oyster species.

KEY WORDS: *Pteria penguin*, gametogenic cycle, pearl oyster

INTRODUCTION

There are many species of pearl oyster in Thailand. Among these species only 3 species, *Pinctada maxima*, *Pinctada fucata*, and *Pteria penguin*, are widely cultivated in Phuket Island, Phuket Province, and Samed Island, Rayong Province. *P. maxima* produce large and good quality pearls. They are widely cultured to produce round pearls, which are quite expensive. *P. fucata* are smaller in size and they are cultured to produce small round pearls. *P. penguin* are medium in terms of size and are cultured to produce hemispherical pearls.

Pearl culture presents a significant potential for economic development in coastal village communities throughout the range of the more valuable species. The industry requires minimal capital input, yet has wide ranging benefits to farmers, coastal communities, and national economies. Pearls are the ideal export commodity; they are nonperishable, shipping costs are negligible, and lucrative markets are already established.

The biology of pearl oysters is poorly understood considering the importance of pearl culture and shell fisheries (Chellem 1987, Gervis & Sims 1992). Research and development priorities in developing countries include the assessment and protection of remaining stocks, evaluation of culture potential, and definition of management strategies for disease prevention. Improvements in spat collection methods, recent hatchery culture successes, selective breeding and genetic manipulation, and advances in pearl implantation techniques all have potential applications in village-based production (Alagarwami et al. 1987).

Among pearl cultures in Thailand, only that of *P. maxima* has been extensively studied, especially in regard to obtaining spat. They are the most important species in the pearling industry of Thailand. For the other two species, they are either collected from the sea or their spats are imported from Japan. There has been an attempt to culture *P. penguin* at Phuket Island but it is still at the early stage. In addition, information on the reproductive biology

such as gametogenesis and reproductive cycles of these three species of pearl oyster is still lacking in Thailand. Because *P. penguin* can be easily collected by SCUBA diving, this species was chosen to study gametogenesis and the reproductive cycle.

MATERIALS AND METHODS

Ten to 15 *P. penguin* pearl oysters were collected monthly between January 2000 and January 2001 from natural stock in the Si-chang Islands, Chonburi Province. Specimens were collected by SCUBA diving at 15–18 m depth. The pearl oysters were measured for shell length and shell width with Vernier calipers and the soft body weights were recorded. Water temperature and salinity at the collecting site were recorded.

A total of 142 *P. penguin* pearl oysters (72 males and 70 females) were examined histologically to determine the gametogenic cycle of both sexes. Gonad tissue between the proximal end of the gut loop and the base of the foot was excised to obtain the largest sections possible (Rose et al. 1990, Wada et al. 1995). Samples were fixed in Bouin's solution overnight. The tissues were then washed in 70% ethyl alcohol, dehydrated with a graded series of ethyl alcohol (70% to 100%) for 30 minute each, embedded in paraffin and sectioned and stained with Harris hematoxylin-eosin or PAS hematoxylin. Sections were observed and photographed under an Olympus CH40 light microscope.

In addition, samples of gonads were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.8) for 24 hours. The tissues were then washed in the buffer solution and postfixed for 1 hour in 1% osmium tetroxide in 0.1 M buffer. After being dehydrated in a graded series of ethyl alcohol, the tissues were embedded in Araldite 502 resin. Sections (1 μ m) were stained with 1% methylene blue and observed under an Olympus CH40 light microscope.

RESULTS

The water temperature and salinity at the collecting site of pearl oyster, *P. penguin* were measured every month. The water tem-

*Corresponding author. E-mail: scmkt@mahidol.ac.th

perature was not much different all year round being in the range of 26.8–30°C. The salinity at the collecting site was quite stable all year round (30–32 ppm).

In general, the female *P. penguin* shells were slightly larger than those of males. The mean shell width and length were 14.59 ± 1.69 cm and 11.32 ± 1.34 cm, respectively. Male and female *P. penguin* could not be distinguished by their shell morphology. Gonad color was also an unreliable indicator of sex. The gonads of *P. penguin* were not discrete organs, but were a greater proliferation of gonad follicles in the area between the byssal gland and gut loop. Gonad follicles proliferate within the connective tissue between the epithelium and the visceral mass.

Classification of Germ Cells

Germ cells appearing in the histologic sections of gonads of *P. penguin*, as observed by light microscope in paraffin and plastic-embedded semithin sections, could be classified as follows:

1. Spermatogenesis

The testis of *P. penguin* is composed of several acini, each surrounded by connective tissue. Based on nuclear characteristic and cell size, the male germ cells of *P. penguin* can be classified into 10 stages. They are spermatogonium, 5 stages of primary spermatocyte, secondary spermatocyte, 2 stages of spermatid and spermatozoa (Fig. 1).

Spermatogonium (Sg). The spermatogonia are attached largely to the acinus wall. The cell is spherical or oval shaped with nuclear size about 5 μ m in diameter (Fig. 1A). The nucleus contains mostly euchromatin and small blocks of heterochromatin dispersed throughout (Fig. 1A). The thin cytoplasm was stained light blue with methylene blue and the nucleolus is distinguishable within the nucleus.

Primary spermatocyte (PrSc). The primary spermatocyte consists of 5 stages: leptotene (LSc), zygotene (ZSc), pachytene (PSc), diplotene (DSc) and metaphase (MSc) (Figs. 1 A, B, C). The distinctive differences among various PrSc are the chromatin condensation pattern and the amount of euchromatin and heterochromatin.

Leptotene spermatocyte (LSc). The spherical-shaped cell of LSc is larger than Sg with a nuclear size approximately 5 μ m in diameter (Fig. 1B). The chromosome begins to condense into small blocks of heterochromatin and are scattered throughout the nucleus (Fig. 1B). The nucleolus is still present.

Zygotene spermatocyte (ZSc). The cell in this stage is approximately the same size as LSc (nuclear diameter of 5 μ m) (Fig. 1C). The heterochromatin blocks become larger and increase in thickness (Fig. 1C). The nucleolus is no longer distinct.

Pachytene spermatocyte (PSc). The PSc cell is smaller than ZSc (Figs. 1A, C). The nucleus contains mostly heterochromatin which are long thick fibers and are arranged in bouquet-like pattern (Figs. 1A, C). The nucleolus is not visible.

Diplotene spermatocyte (DSc). The nuclear size of DSc is about 4 μ m in diameter. The chromatin strands become increasingly condensed and are more visible than those of the earlier stages (Fig. 1A).

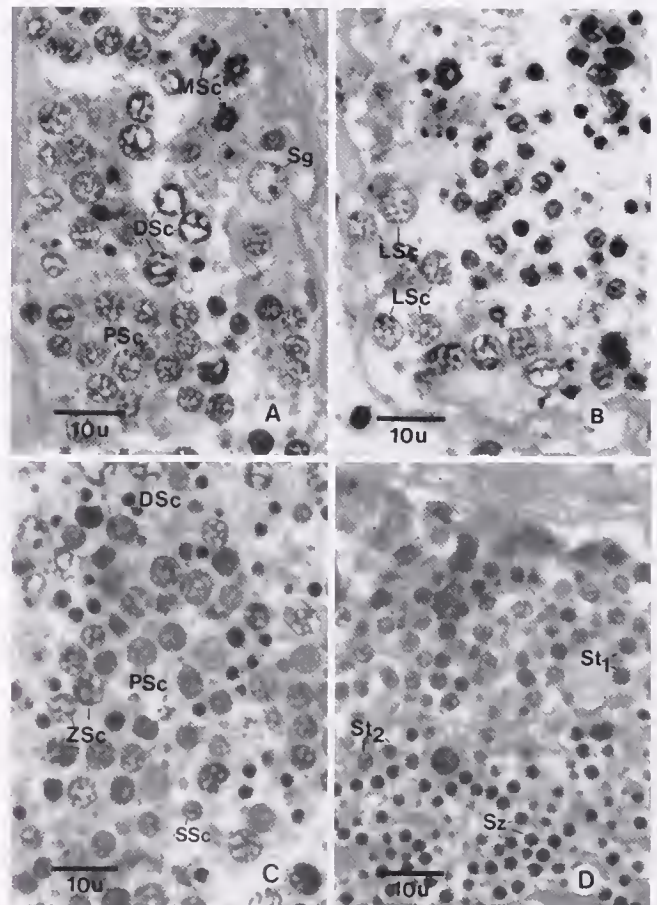


Figure 1. Semithin sections of the testis demonstrating various stages of male germ cells. (A–C) The spermatogonia (Sg) and different stages of primary spermatocyte (PrSc) are lying close to the follicular wall. LSc, leptotene spermatocyte; ZSc, zygotene spermatocyte; PSc, pachytene spermatocyte; DSc, diplotene spermatocyte; MSc, metaphase spermatocyte; SSc, secondary spermatocyte. (D) Spermatids 1,2 ($St_{1,2}$).

Metaphase spermatocyte (MSc). The cell in this stage displays the condensed chromatin (Fig. 1A). The nuclear size is similar to those of the previous stages. The thick chromatin strands move to align along the center of the cell (Fig. 1A).

Secondary spermatocyte (SSc). SSc is a round cell. The thick chromatin blocks are clumped along the cell periphery (Fig. 1C).

Spermatid. Spermatid consists of spermatid I and II.

Spermatid I (St_1). The cell becomes smaller than SSc (Fig. 1D). Its nucleus is spherical and about 1.5–2 μ m in diameter and is located close to the lumen. The chromatin appears as small, thin clumps throughout the cell (Fig. 1D).

Spermatid II (St_2). The general features of St_2 are similar to those of St_1 but the cell size is decreased and the chromatin becomes completely condensed (Fig. 1D).

Spermatozoa (Sz). The cell is spherical with 1 μ m in diameter. The nucleus contains dense chromatin (Fig. 1D). The acrosome is not visible in semithin section. The heads of spermatozoa are separated from trabeculae and aligned in rows (Fig. 1D).

2. Oogenesis

The ovary of *P. penguin* is composed of several acini enclosed by trabeculae or connective tissue. Early-staged oocytes are attached to the trabeculae and mature oocytes are located in the lumens of acini (Fig. 2B). There are 6 stages of female germ cells of *P. penguin* based on cell size and shape. They are the oogonia and 5 stages of oocyte (Fig. 2).

Oogonium (Og). The oogonium is round in shape (Figs. 2A, B). The nuclear envelope is not distinct. Og is about $3.5\ \mu\text{m}$ in size and contains mostly euchromatin (Fig. 2A). The cells are attached to the inner side of trabeculae (Fig. 2A).

Oocyte I (Oc₁). Oc₁ is about $14\ \mu\text{m}$ in size. The nucleus is round and about $8\ \mu\text{m}$ in diameter (Figs. 2A, B). It contains mostly euchromatin, which is dispersed throughout the nucleus. The nucleolus is clearly present (Figs. 2A, B). The Oc₁ cells are attached to the trabeculae (Fig. 2B).

Oocyte II (Oc₂). Oc₂ contains a large nucleus and a thin cytoplasm (Fig. 2B). The cell size is about $18 \times 22\ \mu\text{m}$, with the

nuclear size about $11 \times 12\ \mu\text{m}$. The nucleus contains a distinct nucleolus (Fig. 2B). Lipid droplets are present in the cytoplasm. Oc₂ are still attached to the trabeculae (Fig. 2B).

Oocyte III (Oc₃). The cell size is about $25 \times 31\ \mu\text{m}$, with the nuclear size being about $15 \times 17\ \mu\text{m}$. The cell is larger in size and the nucleus contains mostly euchromatin (Figs. 2B, D). There are numerous yolk granules, which are dispersed throughout the cytoplasm. Oc₃ cells still remain attached to the trabeculae at the base of the cell (Figs. 2B, D).

Oocyte IV (Oc₄). The cell is large and it assumes the droplet shape (Figs. 2C, D). The cell size is about $31 \times 51\ \mu\text{m}$, with the nuclear size being about $22 \times 26\ \mu\text{m}$. The nucleolus is clearly visible and the nucleus occupies most of the cell area (Fig. 2C). The ratio of cytoplasm to nucleus is about 0.44. Yolk granules are numerous. The Oc₄ cell is covered with a very thin jelly coat on the outer cell membrane (Figs. 2C, D). The cells are still attached to the trabeculae (Fig. 2D).

Oocyte V (Oc₅). This is the mature oocyte before spawning. Oc₅ is the largest cell and has a polygonal or round shape (Fig. 2D). The cell size is about $42\ \mu\text{m}$ in diameter and the nuclear size is about $27\ \mu\text{m}$ in diameter. The nucleus is very large when compared with that of Oc₄. The ratio of cytoplasm to nucleus is 0.56. Yolk granules are abundant. All Oc₅ cells are completely detached from the trabeculae but remain in the capsule (Fig. 2D).

Reproductive Cycle

The reproductive cycle of *P. penguin* was studied by observing the changes in the gonad histology. The stages of gonad maturation could be classified into 3 stages in the male (developing, mature, partial spawning) and 4 stages in the female (developing, mature, partial spawning, spent).

1. Male

Developing stage. The gonad was characterized by the expansion of the follicle and the appearance of spermatogonia along the follicular wall (Fig. 3A). Primary spermatocytes proliferate rapidly and move to the center of the follicular lumen (Fig. 3B). The spermatids and spermatozoa are present in the lumen but they are not abundant (Fig. 3B). The developing stage of the male occurred all year round but production of spermatocytes was highest in August and November (Fig. 5).

Mature stage. The gonad still contains early staged cells but they are attached to the wall like a narrow band. The spermatid and spermatozoa, which occupy most areas of the follicle, are radiating toward the center of the follicle. They arrange themselves in radial rows (Fig. 3C). The mature stage of male *P. penguin* occurred from February to March during the annual spermatogenic cycle (Fig. 5).

Partial spawning stage. This is the stage when the pearl oysters release mature gametes into the water. The gonad contains a few spermatozoa in the lumen and the follicular wall is wrinkled and partially collapsed (Fig. 3D). Partial spawning stage occurred twice a year, in January and April (Fig. 5).

2. Female

Developing stage. The ovary consisted of capsule-like structures, which mostly contained early stage oocytes. Og, Oc₁, Oc₂,

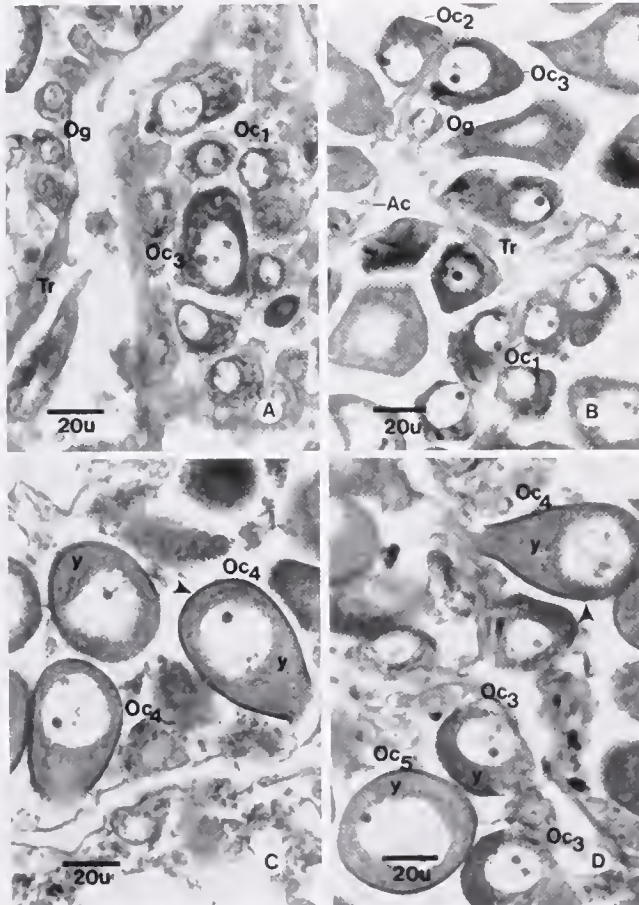


Figure 2. Paraffin section of the ovary showing the oogenic unit, which consists of several acini (Ac). (A, B) Early stage oocyte, oogonium (Og), oocyte I (Oc₁), oocyte 2 (Oc₂), and oocyte 3 (Oc₃), are attached to the trabeculae (Tr). (C, D). The mature oocytes, oocyte 4 (Oc₄) and oocyte 5 (Oc₅), are located in the lumen of the acinns. The mature oocyte exhibits basophilic cytoplasm. Yolk granules (y) are dispersed throughout the cytoplasm and the thin jelly coat (arrow heads) is clearly present.

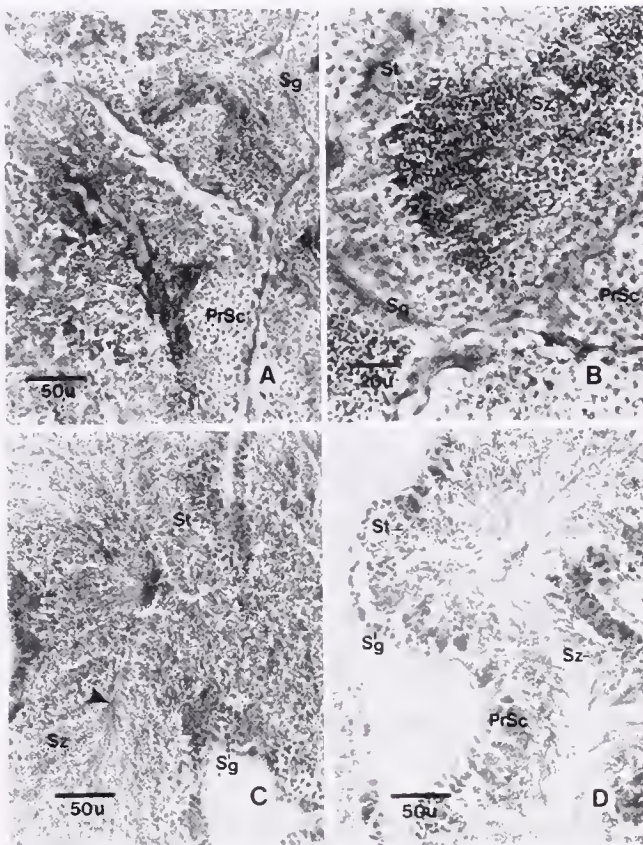


Figure 3. Paraffin sections of the testis showing various stages in the reproductive cycle. (A, B) the developing stage showing follicles that contain spermatogonia (Sg), primary spermatocyte (PrSc), spermatids (St), and spermatozoa (Sz). Spermatogonia are attached to the follicular wall and primary spermatocytes are located next to the spermatogonia on the inner side of the acini. (C) The mature stage contains mostly late spermatids and numerous spermatozoa, which lie in rows. The tails of spermatozoa are present in the center of the follicular lumen (arrow head). (D) Partial spawning stage showing partly empty follicles. The testis contains a few spermatozoa (Sz), spermatids (St) and primary spermatocytes (PrSc).

and Oc₃ (Fig. 4A). These oocytes were attached to the trabeculae (Fig. 4B). The developing stage occurred from August to December (Fig. 6).

Mature stage. In the mature ovary, there are abundant Oc₄ and Oc₅, but only a few remaining early staged oocytes. Most oocytes have a polygonal or a drop shape, and display both nucleolus and nucleus (Fig. 4B). Some are attached to the trabeculae but most oocytes are free in the center of the acinus (Fig. 4B). The mature stage of the female occurred in January, August to September, and December (Fig. 6).

Partial spawning stage. The follicles of the ovary appear partially empty, indicating that some mature oocytes have been released (Fig. 4C). The follicles contain mostly Oc₄ and Oc₅ that have become rounded, polygonal, or pear shaped (Fig. 4C). The early-stage oocytes are still attached to the trabeculae (Fig. 4C). Partial spawning occurred in March and July (Fig. 6).

Spent stage. Mature oocytes are completely discharged and the follicles are almost empty (Fig. 4D). The follicular wall be-

comes wrinkled or degenerates and the gonads are greatly decreased in size (Fig. 4D). The spent stage of female *P. penguin* occurred in November and December (Fig. 6).

DISCUSSION

The characteristics of gametogenesis and stages of gonad development in *P. penguin* are similar to those described for the pearl oysters *P. albina* (Tranter 1958a), *P. margaritifera* (Tranter 1958c), *P. mazatlanica* (García-Domínguez et al. 1996), *P. fucata* (Tranter 1959, Wada et al. 1995), and *P. maxima* (Rose et al. 1990). However, previous authors did not emphasize the classification of germ cells. In *P. maxima* and *P. fucata fucata*, spermatogenic cells were classified into 4 stages: spermatogonia, spermatocyte, spermatid, and spermatozoa (Rose et al. 1990, Wada et al. 1995). In *P. albina*, Tranter (1958b) categorized male germ cells into 5 stages. Thielley et al. (1993) studied the ultrastructure of spermatogenic cells in *P. margaritifera* and classified them into 10 stages. Based on cell size and nuclear characteristics, the present study revealed 10 stages of spermatogenic cells: spermatogonia, 5 stages of primary spermatocyte, secondary spermatocyte, 2 stages

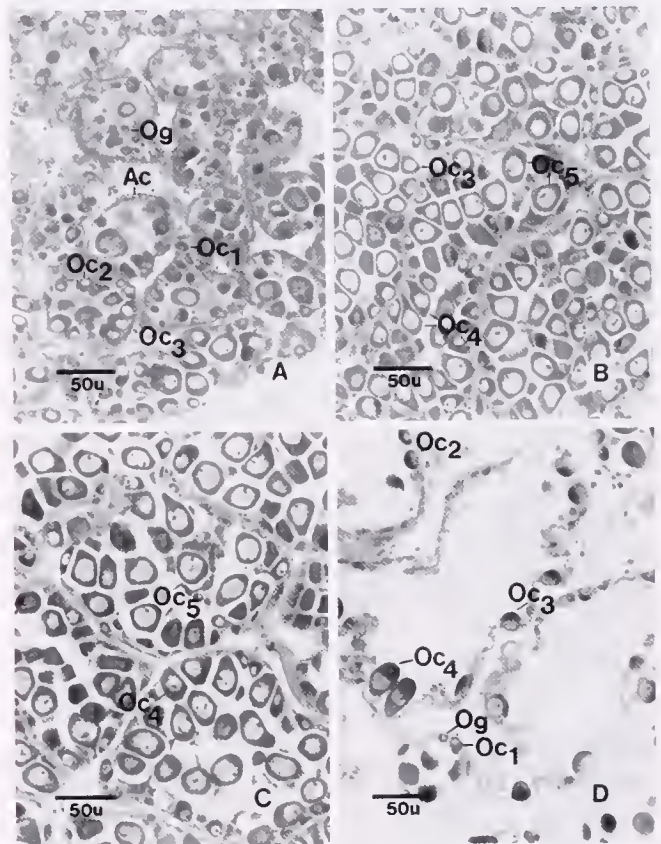


Figure 4. Paraffin sections of the ovary showing the female reproductive cycle. (A) The developing stage mainly contains early stage oocytes, oogonia (Og) and oocytes stage 1–3 (Oc_{1–3}) in the acini (Ac). (B) The mature stage mostly contains a few early stage oocytes (Oc_{1–3}), and late stage oocytes (Oc_{4–5}) that have increased in size and number. (C) Partial spawning stage showing partly emptied follicles. The ovary contains a few early stage oocytes and the mature oocytes (Oc₄, Oc₅) are free in the follicular lumen. (D) The spent stage showing the empty follicles after the discharge of mature oocytes. There are still some oogonia (Og) and oocytes (Og, Oc_{1–4}) in the follicle.

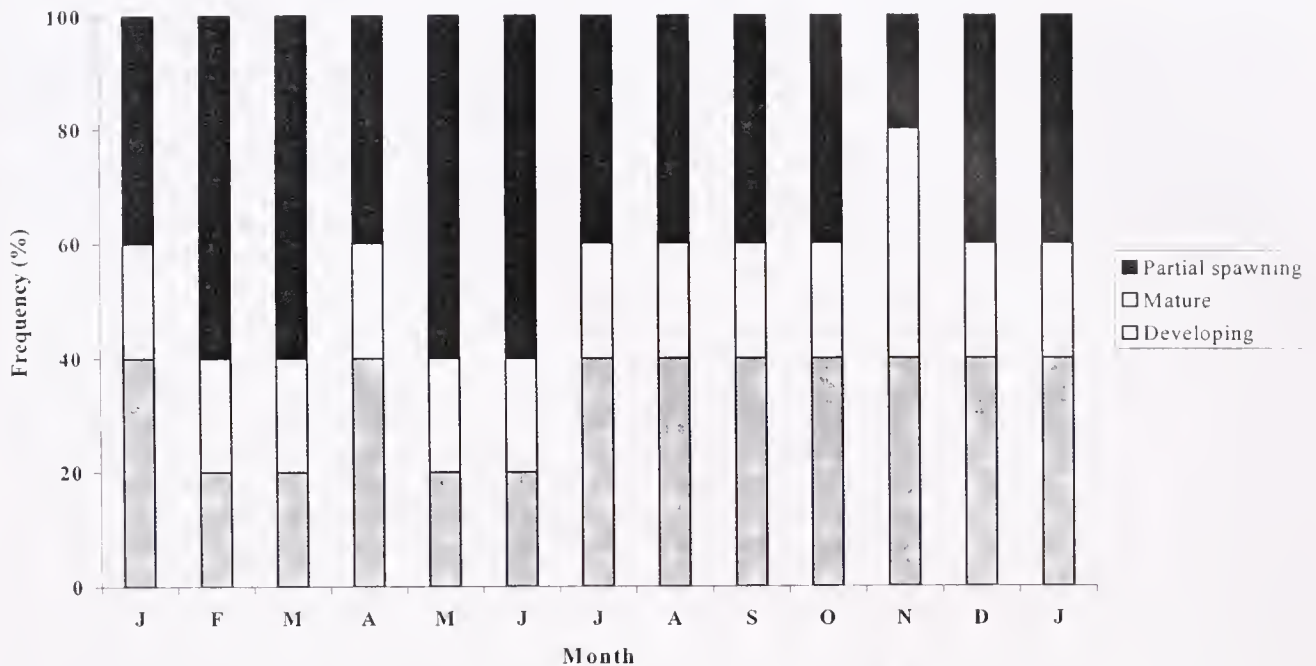


Figure 5. The annual spermatogenic cycle of *P. penguin* from monthly collections between January 2000 and January 2001.

of spermatid, and spermatozoa. However, in this study the acrosome could not be seen on the anterior end at high magnifications, which was different from that reported for *P. albina* (Tranter 1958b) and the primary and secondary spermatogonia could not be distinguished like those found in *P. margaritifera* (Thielley et al. 1993). In addition, diaknetic primary spermatocytes were not observed; only metaphase primary spermatocytes were found. This study identified 2 stages of spermatid based on nuclear size and chromatin condensation, but in *P. margaritifera*, only one stage of spermatid was found.

The spermatogonium is the cell whose nucleus contains a single nucleolus and small clumps of dense chromatin. The spermatogonium divides into the first meiotic division to give rise to the primary spermatocyte. Prophase cells exhibit a different form of chromatin condensation. The chromatin appears as a very dense cluster in the leptotene spermatocyte. The zygotene spermatocyte is characterized by the presence of a synaptonemal complex in *P. margaritifera* (Thielley et al. 1993). However, a synaptonemal complex could not be identified in the zygotene spermatocyte of *P. penguin* in this study. In the secondary spermatocyte, the thick

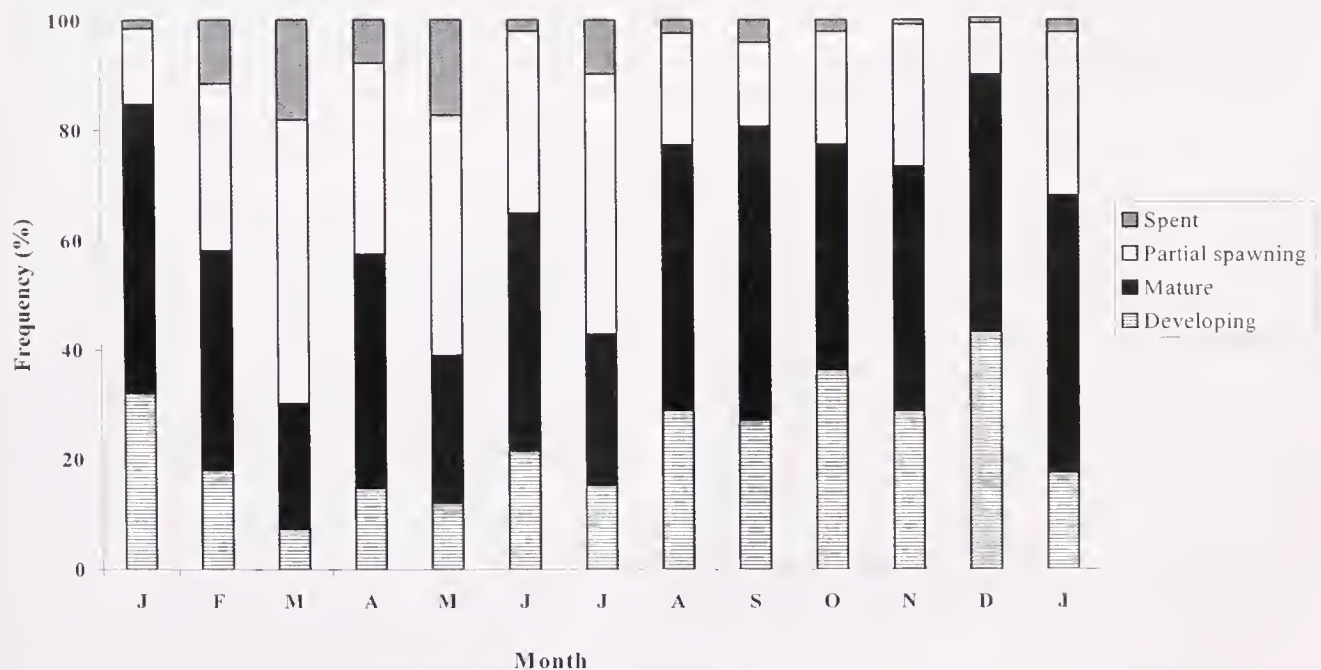


Figure 6. The annual oogenic cycle of *P. penguin* from monthly collection between January 2000 and January 2001.

chromatids are clumped along the cell periphery. Spermatids are classified into spermatid I and spermatid II, whose chromatins become condensed throughout spermatogenesis. During the process, the few clear areas between chromatin masses are reduced in size (Thielley et al. 1993). Baccetti and Afzelius (1976) described 4 main types of sperm: primitive, modified, biflagellate, and aflagellate. Most studies have shown that bivalves have "primitive spermatozoa" (Dohmen 1983). The spermatozoa of *P. penguin* is of the primitive type, similar to those of other bivalves. Primitive sperms are typical of animals that spawn their gametes into surrounding water.

Literature on oogenesis of pearl oysters in the Family Pteriidae is very scarce, especially on *Pteria* species. Various stages of oogenesis have been described along with reproductive cycles in *P. maxima* and *P. fucata fucata* (Rose et al. 1990, Wada et al. 1995). *P. maxima* has 4 stages of oogenesis (Rose et al. 1990), whereas 3 stages were identified in *P. fucata fucata* (Wada et al. 1995). According to Tranter (1958b), oogenesis of *P. albina* occurred in 6 stages. This study identified 6 stages of oogenesis in *P. penguin* (oogonia, 5 stages of oocytes Oc₁₋₅) based on cell size, nuclear size, basophilic cytoplasm, presence of yolk granules, and jelly coat. These oogonia are in the previtellogenic phase, which is characterized by the growth of oocytes and an increase in volume of the nucleus and cytoplasm (Dohmen 1983).

Oc₃₋₅ in *P. penguin* could be classified into the vitellogenic phase. During this period the oocytes grow rapidly, mainly because of the accumulation of yolk and other nutrient substances such as glycogen and lipid (Dohmen 1983). The cytoplasm increased in size due to the presence of yolk granules. This study did not identify glycogen or lipid droplets, but the yolk granules were quite distinct.

Similar to *P. albina* (Tranter 1958b), Oc₄ are larger in size and the cytoplasm stains more heavily as yolk accumulates. Besides the presence of yolk granules, both Oc₄ and Oc₅ are surrounded by a thin jelly coat, which stained positively with PAS, indicating that it is mucopolysaccharide in nature. Oc₅ could be distinguished from Oc₄ by the larger nucleus and detachment from the trabeculae (Tranter 1958b).

Histological examinations of the ovarian and testicular tissues of *P. penguin* showed a definite annual reproductive cycle. Previ-

ous studies simplified the gametogenic cycle of family Pteriidae into 5 to 7 stages, which are clearly defined in females (Rose et al. 1990, Wada et al. 1995, Pouvreau et al. 2000). In this study, histological examination determined 3 stages in the male and 4 stages in the female. In the male, they are the developing, mature, and partial spawning stages. Spent or completely empty follicles of the testis were not observed in the male *P. penguin*. In the female, gonad development stages are developing, mature, partial spawning, and spent stages. In general, the gametogenic cycle is clearly defined in female *P. penguin*. The present study could only identify the main stages as described previously.

The histological examination of *P. penguin* showed that there were peaks of partial spawning in the male that occurred in March to June (summer) and December to January (winter). In the female, partial spawning occurred in January to May (winter to summer). A similar reproductive cycle has been reported in *P. fucata fucata* in the Gulf of Mannar, Sri Lanka. The spawning season was reported to be in the midsummer and midwinter (May to July, November to January) with continuous breeding (Pearson et al. 1929). But the breeding seasons of each of these species also differ.

Most studies reported a wide range of water temperature all year round, e.g., 18°C to 32°C in Western Australia (Rose et al. 1990), and 11°C to 29°C in Japan (Wada et al. 1995). Rose et al. (1990) considered variations in reproductive cycle to be due to the temperature range in different locations as influenced by latitudinal position. Other bivalve species exhibit distinct seasonal reproductive cycle, which usually are related to temporal variations of environmental factors such as food availability, water temperature, and/or photoperiod. In this study, the water temperature and salinity at the collecting site were quite similar all year round. It is possible that the lack of a highly defined reproductive or gametogenic cycle in *P. penguin* is related to the lack of observed temperature and salinity fluctuations.

ACKNOWLEDGMENTS

This investigation was supported by the Post Graduate Education, Training and Research Program in Environmental Science, Technology, and Management under Higher Education Development Project of the Ministry of University Affairs, Bangkok, Thailand.

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MICROSCOPIC OBSERVATIONS OF LARVAL *OSTREA CIRCUMPICTA* (BIVALVE: OSTREIDAE) IN BROOD CHAMBERS

DO-HYUNG KANG,¹ SE-JAE KIM² AND KWANG-SIK CHOI^{1*}

¹School of Applied Marine Science, College of Ocean Science, Cheju National University, 1 Ara I-Dong Jeju 690-756, Republic of Korea; ²Department of Life Science, Cheju National University, Republic of Korea

ABSTRACT This study is the first report of the microscopic features of larvae and gonads of *Ostrea circumpecta* collected in Jeju, Korea. Female oysters brooded larvae at the early veliger stage (115–135 μ m) in the infrabranchial chamber. Larvae in this chamber displayed a well-developed velum covered with long and short cilia; the adductor muscle was incompletely developed. The digestive system of the larvae, including the mouth, esophagus, stomach, digestive gland, and intestine, was identifiable in histologic preparations, indicating that the veliger larvae in the brood chamber were capable of feeding using the velum and cilia. Diameters of ripe eggs in the ovary ranged from 67–120 μ m. Most female oysters collected in June 2001 were spawning or absorbing whereas most of male oysters were spent or sexually inactive.

KEY WORDS: ostreidae, *Ostrea circumpecta*, reproduction, larval morphology, brood oyster, Korea

INTRODUCTION

Oysters of the genus *Ostrea* are brooders, releasing larvae instead of gametes during spawning (Buroker 1985, Ó Foighil & Taylor 2000). Gametes of brood oysters are fertilized at the ctenidia, and the embryos are released into the branchial chamber of females. In this chamber, the larvae undergo pelagic development, including trochophore and veliger stages (Strathmann & Strathmann 1982, Mackie 1984, Ó Foighil & Taylor 2000). Veliger larvae in the chamber are characterized by velum covered with cilia that are used for locomotion and feeding (Elston 1999, Chaparro et al. 1999 & 2002). Swimming veliger larvae are often observed in suprabranchial or infrabranchial chambers (Chaparro et al. 1993). However, brooding oyster larvae are often kept in interlamellar spaces or brood sacs and are immobile (Mackie 1984, Tankersley & Dimock 1992).

Microscopic examination of larvae provides valuable information on feeding type, developmental mode, planktonic period, and larval settlement (Strathmann & Leise 1979, Pechenik 1986, Strathmann et al. 1993, Chaparro et al. 1999). Histology has been widely and extensively used to examine the larval structure and development of marine bivalves (Howard & Smith 1983, Elston 1999). In addition, video cameras and endoscopes are often used in microscopic observations of marine bivalve larvae (Ward et al. 1991, Strathmann et al. 1993, Tankersley & Dimock 1993, Bosch & Slattery 1999, Elston 1980, Chaparro et al. 1999&2002).

Ostrea circumpecta is abundant on rocky intertidal to subtidal zones of Jeju Island (Kwon et al. 1993), Korea, where salinity remains high and stable all year (Lee et al. 2000). Like other oysters of the genus *Ostrea*, this species is a brooder and is widely distributed in southern Japan, China, and Korea (Hirase 1930, Bernard et al. 1993, Kwon et al. 1993). Despite its abundance, few studies have investigated the ecology and reproductive biology of *O. circumpecta* (Bae & Bae 1972, Yang 1999&2001). Here, we provide the first report of the microscopic features of the gonads and larvae of *O. circumpecta* in sexually mature individuals.

MATERIALS AND METHODS

Munseom, an uninhabited island located off the southern coast of Jeju (33°13' 25"N, 126°33' 58"E), is well known for its high species diversity and richness of marine biota (Je et al. 2002, Fig. 1). The island characterized by volcanic rocky intertidal and subtidal zones that are subjected to strong wave action. The rocky substrata are enriched with sessile fauna such as oysters, barnacles, and numerous species of anthozoans. In particular, *O. circumpecta* heavily encrusts the subtidal cliff at depths between 3 and 6 m. Surface water temperatures and salinity in this area vary from 16 °C to 22 °C and from 32.2–34.4 psu annually (Choa & Lee 2000, Lee et al. 2000). Due to the influence of the warm Kuroshio current, surface water temperatures during winter are several degrees higher than in northern Jeju.

O. circumpecta were collected in June 2003 by SCUBA diving at depths between 3 and 5 m. Oyster tissues were fixed in Davidson's fixative, and longitudinal sections were made along the middle of the body of fixed specimens (Fig. 2A) using standard histologic methods. Microscopic images of oyster eggs and larvae in brood chambers were converted to digital image using a digital camera installed on the microscope. The size of eggs and larvae were then measured from the images using image analysis software (Kang et al. 2003).

RESULTS

Figure 2 shows a female *O. circumpecta* collected in June 2003. This species has a relatively large adductor muscle and gills compared with nonbrooding oysters. The mantle is composed of two layers with thick mantle lobes. We found no hermaphroditic individuals in this study. Figure 3 shows numerous early veliger larvae in the infrabranchial chamber. The visceral masses of the larvae were filled with coelomic fluid in the coelomic cavity. The larvae were approximately 115–135 μ m long, and we observed no physical connections between female oysters and larvae. No obvious structural changes for holding larvae were found in the gills. Figure 4 shows intact larvae isolated from the chamber. The upper part of the velum was covered with two types of cilia: long outer preoral cilia (i.e., OPC, Chaparro et al. 1999), 20–40 μ m long and short inner preoral cilia (IPC), 5–10 μ m long.

*Corresponding author. E-mail: skchoi@cheju.ac.kr

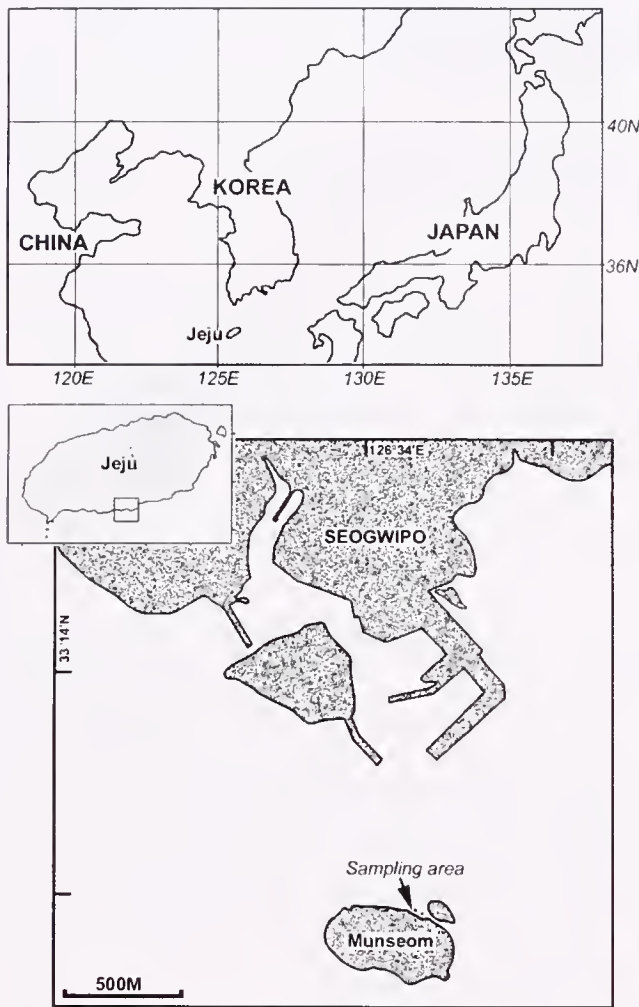


Figure 1. Sampling location.

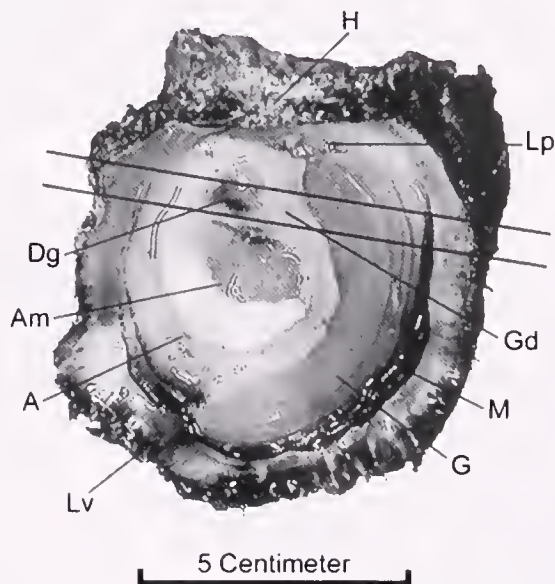


Figure 2. *O. circumpicta*. General morphology of an adult. Bold parallel lines in the middle of the body show the location where the tissue for histologic cross-section was collected.

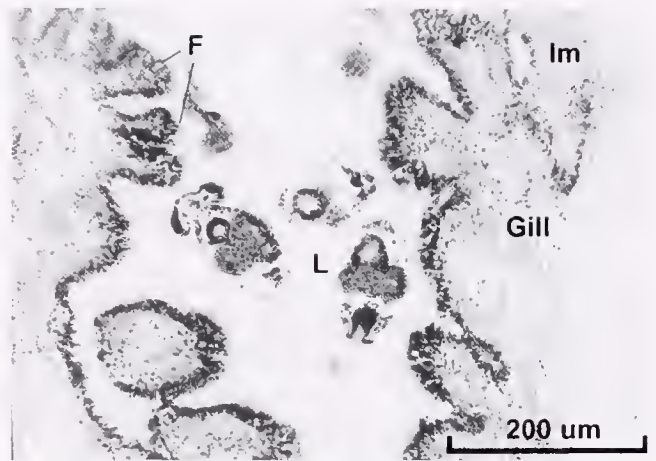


Figure 3. Photomicrograph of larvae in the infrabranchial chamber of *O. circumpicta*. F: filament; L: larvae; Im: interlamellar muscle.

Figures 5 and 6 illustrate the internal structure of early veliger larvae. The mouth is located at the edge of the lower part of the velum, and the oral opening lead to the esophagus. The stomach is visible at the end of the digestive tract. The larval stomach, digestive gland, and intestine form the digestive system. Although it is not completely developed, the adductor muscle is identifiable in the larvae. Cilia are mainly located on the periphery of the velum; sensory cilia are also found on the apical plate located at the center of the velum. Some particles were identified in the digestive gland that were believed to be ingested diatoms (Kohn, personal communication).

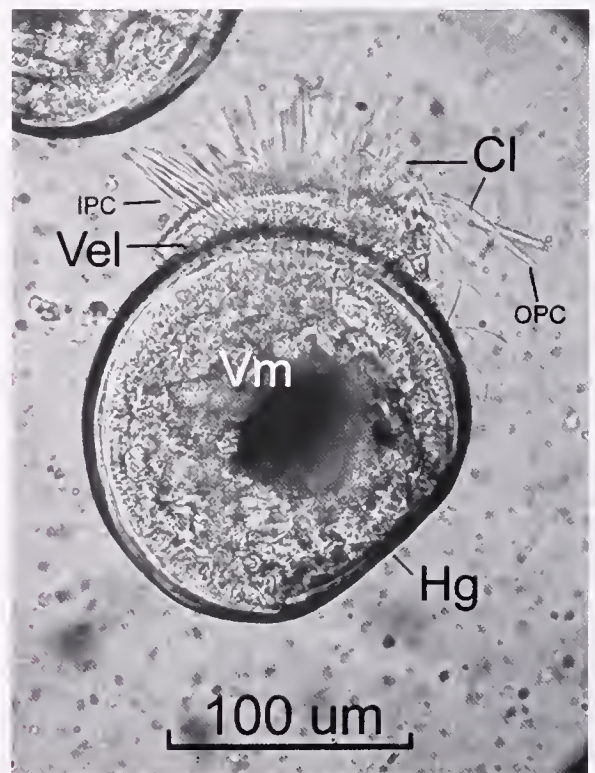


Figure 4. Early *O. circumpicta* veliger showing the organs. Cl, cilia; Hg, hinge; IPC, inner preoral cilia; OPC, outer preoral cilia; Vel, velum; Vm, visceral mass.

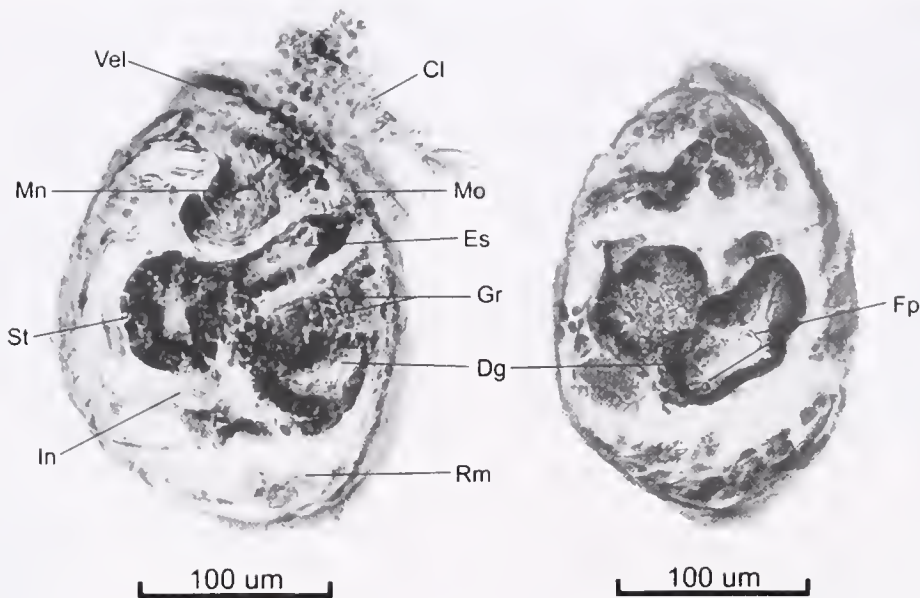


Figure 5. Longitudinal section of larval *O. circumpicta*. Cl, cilia; Dg, digestive gland; Es, esophagus; Fp, food-like particle; Gr, gill rudiment; In, intestine; Mo, mouth; Mn, mantle; Rm, retractor muscle; St, stomach; Vel, velum.

Figure 7 shows a longitudinal section of female oyster whose follicles are packed with ripe eggs (see Fig. 7A). The diameter of egg nuclei ranged from 16–35 μm , with a mean of 26 μm ($n = 29$). Egg diameter ranged from 67–120 μm , with a mean of 89 μm ($n = 60$). However, most ripe eggs were ready for discharging from the follicles 90–110 μm in diameter. Some degenerating ova

were surrounded by hemocytes, indicating that the eggs are phagocytosed by hemocytes (see Fig. 7B). Nine male oysters analyzed in this study exhibited spent testes or were in sexually inactive (see Figs. 7C, 7D), indicating that males spawned earlier than females.

DISCUSSION

In marine bivalves, fertilization and development of larvae is either external or internal (Sastry 1979, Strathmann & Strathmann 1982, Olive 1985, Levitan & Petersen 1995). Oysters of the genus *Crassostrea* broadcast eggs and sperm in the water column, and fertilization and subsequent larval development occurs externally. In contrast, in oysters of the genus *Ostrea*, egg fertilization takes place internally in the gills (Andrews 1979, Harry 1985, Chaparro et al. 1993, Ó Foighil & Taylor 2000). The embryos or larvae are then raised in the mantle cavity, suprabranchial, or infrabranchial chamber of the female (Buroker 1985, Chaparro et al. 1993, Ó Foighil & Taylor 2000). In this study, larvae were located mostly in the infrabranchial chamber (see Figs. 2, 3), indicating that *O. circumpicta* uses its gills as a brooding chamber, as has been observed in other brooding oysters of the genus *Ostrea*.

Early veliger larvae located in the infrabranchial chamber showed a well-developed velum covered with cilia (see Figs. 5, 6). The presence of a ciliary ring around the velum is one of the key characteristics of veliger larvae (Waller 1981, Beauchamp 1986, Strathmann et al. 1993, Chaparro et al. 1999). Cragg (1989) suggested that a well-developed velum with cilia is an adaptation for prolonged planktonic life in the water column. The velum also plays a key role in feeding during the larval period (Hadfield et al. 1997, Klinzing & Pechenik 2000, Chaparro et al. 2002). Larvae in the brood chamber observed in this study exhibited a well-developed digestive system that included the mouth, esophagus, stomach, digestive gland, and intestine (see Fig. 5). Longitudinal section of the larvae also showed some food-like particles in the digestive gland (see Fig. 5), suggesting that the larvae are involved in feeding activities (Elston, 1999). Chaparro et al. (1993) experi-

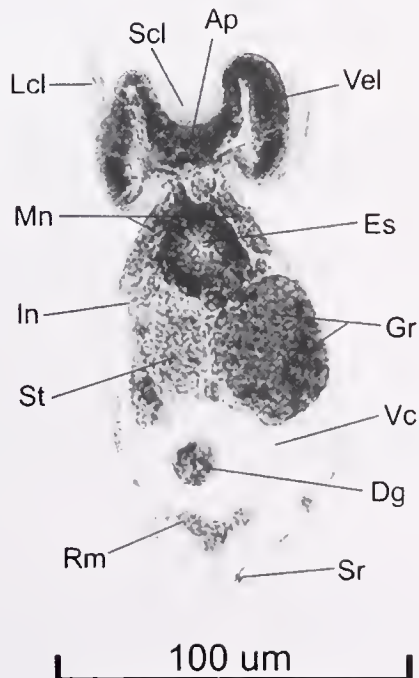


Figure 6. Frontal section of larval *O. circumpicta*. Ap, apical plate; Lcl, locomotory cilia; Dg, digestive gland; Es, esophagus; Gr, gill rudiment; In, intestine; Mn, mantle; Rm, retractor muscle; Scl, sensory cilia; Sr, shell rudiment; St, stomach; Vel, velum; Vc, visceral cavity.

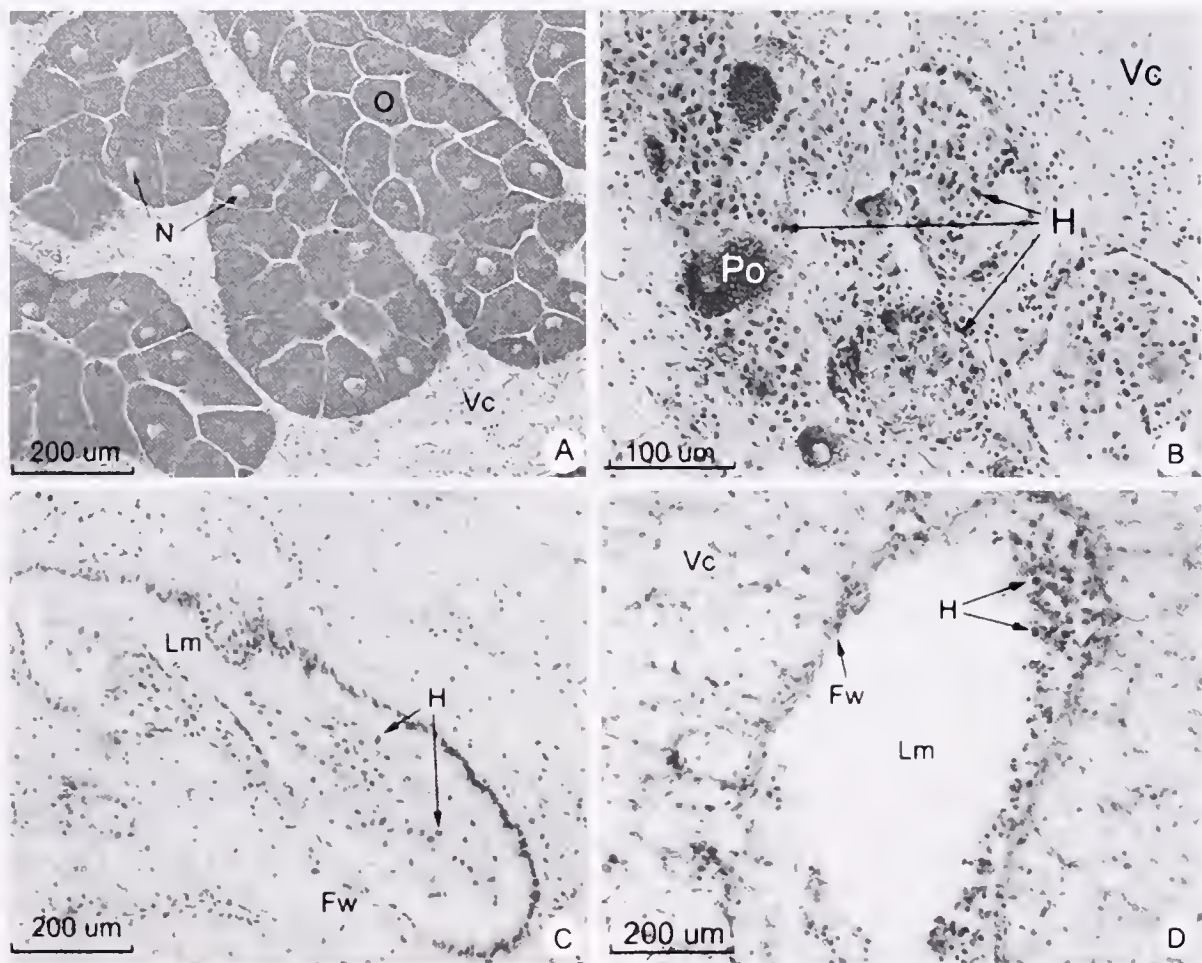


Figure 7. Photomicrograph of *O. circumpicta* gonads. (A) Fully matured ovary showing the ova (O), with nucleus (N), and vesicular cells (Vc). (B) Spent ovary showing phagocytized ova (Po) and phagocytic activities by hemocytes (H). (C, D) Testes of resting stage showing follicle wall (Fw), vacuated lumen (Lm), hemocytes (H), and vesicular cells (Vc).

mentally fed the veliger larvae of *O. chilensis* with plastic particles (2–10 μm in diameter), and the particles were later found in the digestive system. Their study indicated that the veligers were actively involved in feeding activities in the brood chamber. Thus, it is believed that *O. circumpicta* veliger larvae also feed on food particles available in the brood chamber, as was observed in *O. chilensis*.

The size of the *O. circumpicta* egg measured in this study varied from 67–120 μm, which is somewhat larger than the eggs of nonbrooding oysters such as *C. gigas* or *C. virginica* (40–58 μm, Gallagher et al. 1986, Arakawa 1990, Choi et al. 1993, Brousseau 1995, Lango-Reynoso et al. 2000, Kang et al. 2003). The relatively larger egg size of *O. circumpicta* suggests that these oysters produce lecithotrophic eggs, like *O. edulis*, *O. denselamellosa*, or *O. lurida* (DiSalvo et al. 1983, Mackie 1984, Arakawa 1990). DiSalvo et al. (1983) reported that *O. chilensis* larvae in Chilean waters do not ingest food particles when reared outside the brooding chamber, strongly indicating that *O. chilensis* larvae are truly lecithotrophic. In contrast, Chaparro et al. (1993; 1999) used an endoscope to observe veligers of *O. chilensis* (290–300 μm) actively feeding on food particles available in the brood chamber that experimentally provided. The authors concluded that *O. chilensis* larvae are planktotrophic, because they have a complete digestive

system and feed on food particles in the chamber. The *O. circumpicta* larvae observed in this study were 115–135 μm long (i.e., slightly larger than mature eggs [90–110 μm]), indicating that the larvae were in the early veliger stage. Food-like particles found in the digestive system of *O. circumpicta* veliger larvae in this study suggest that the larvae are planktotrophic in the brood chamber.

In conclusion, we observed the larval development of *O. circumpicta* in the infrabranchial chamber using histologic preparations. Ripe and degenerating ova in the gonads and early veliger larvae in brood chamber observed in late June indicated that late June, when water temperature reaches 22 °C, is the spawning. A well-developed velum and digestive system suggested that the larvae are capable of swimming and feeding inside the brood chamber.

ACKNOWLEDGMENTS

The authors thank the staff of shellfish research and aquaculture laboratory of Cheju National University. The authors also thank to Dr. Alan J. Kohn for comments and critique of the manuscript. This work was supported by a grant (No. BDM 0100211) to JRL from the Strategic National R&D Program through the Genetic Resources and Information Network Center funded by the Ministry of Science and Technology, Korea.

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HERPES-LIKE VIRUS ASSOCIATED WITH ERODED GILLS OF THE PACIFIC OYSTER *CRASSOSTREA GIGAS* IN MEXICO

REBECA VÁSQUEZ-YEOMANS,^{1,†} JORGE CÁCERES-MARTÍNEZ,^{1,*†} AND
ANTONIO FIGUERAS HUERTA²

¹Laboratorio de Biología y Patología de Organismos Acuáticos del Departamento de Acuicultura, Centro de Investigación Científica y de Educación Superior de Ensenada, Apdo. Postal 2732, C.P. 22860, Ensenada Baja California, México and ²Laboratorio Nacional de Referencia de enfermedades de Moluscos del Instituto de Investigaciones Marinas de Vigo, Eduardo Cabello #6, 36208, Vigo, España

ABSTRACT Since 1997, high mortality episodes of cultured oysters have occurred in Bahía Falsa, México. Studies on the possible association of these mortalities with pathogens have showed some similar characteristics to those found in the Portuguese oyster *Crassostrea angulata* and the Japanese oyster *Crassostrea gigas* infected by an iridoviridae-like particles in the lately 1960s. This infection was named gill necrosis virus infection (GNV). In a recent study, we could not find any virus using transmission electron microscopy (TEM) in adult oysters with clinical and histologic signs similar to those described for GNV. However, new TEM images showed the presence of viral particles in eroded gills of oysters. Morphologic characteristics, such as thin-walled icosahedric shape, the presence of capsids in an extension of the nucleus or in a vacuole and size varying from 80 to 90 nm suggest that the viruses belong to the Herpes viridae family. This virus could be involved in the mortality episodes in the Bay and it is different to those described as causal agent of GNV.

KEY WORDS: *Crassostrea gigas*, herpes-like virus, mortality, gill erosion, oyster disease

INTRODUCTION

In a recent study on the possible association of high mortality outbreaks of the Pacific oyster *C. gigas* cultured in Baja California and other states of NW Mexico with a pathogenic agent (Cáceres-Martínez & Vasquez-Yeomans 2003), we found some clinical and histopathologic evidence similar to those described for gill necrosis viral infection (GNV) recorded in the Portuguese oyster *C. angulata* and to a lesser degree in the Pacific oyster cultured in France, Great Britain, Spain, and Portugal between 1966 and 1967 (Marteil 1969, Comps 1988). Previously, we did not detect the iridovirus-like the causal agent of GNV, using transmission electron microscopy (TEM), possibly because the sample process, the status of the gill tissues of surviving oysters (wound repair), and/or difficulties in finding the viral agent in fixed tissues (Cáceres-Martínez & Vasquez-Yeomans 2003). However, new TEM analyses of more samples have detected herpes-like virus particles.

MATERIALS AND METHODS

A sampling carried out in November 2000, of 10 adult oysters (Mean 89.3 ± 7.7 mm shell length), from an area of the Bahía Falsa, Baja California ($116^{\circ}00'W$, $30^{\circ}25'N$) where mortalities were common, were reviewed and those showing highly eroded gill tissues (Cáceres-Martínez & Vasquez-Yeomans 2003) were selected for TEM examination. Small pieces of tissue were cut from the eroded area of the gill and fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.8, for 4 h at 4 °C. Fixed tissues were washed for 12 hours at 4 °C in the same buffer and cut into 1-mm³ pieces. These pieces were then postfixed in buffered 1% OsO₄ for 4 hours at 4 °C, dehydrated through a graded series of ethanols and embedded in epoxy resin. Sections (90 nm thickness) were cut and stained with 5% uranyl acetate for 30 minutes,

then observed with TEM operated at 75 kV in the Instituto de Investigaciones Marinas from Vigo, Spain.

RESULTS AND DISCUSSION

Viral particles were found in all samples ($n = 10$) of eroded gill analyzed in this study. The morphologic characteristics of the

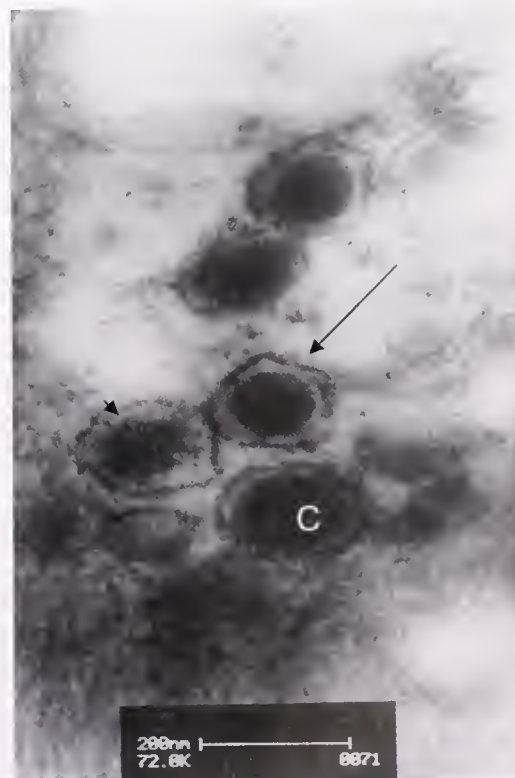


Figure 1. Viral particles showing the pentagonal shape of the capsids (large arrow), thin wall (small arrow), and core (C). Size variations are also observed.

*Corresponding author.

†Present address: Instituto de Sanidad Acuicola, A.C. Calle 9na y Gastelum No. 468 Local 14, Zona Centro. C.P. 22800, Ensenada, Baja California, México.

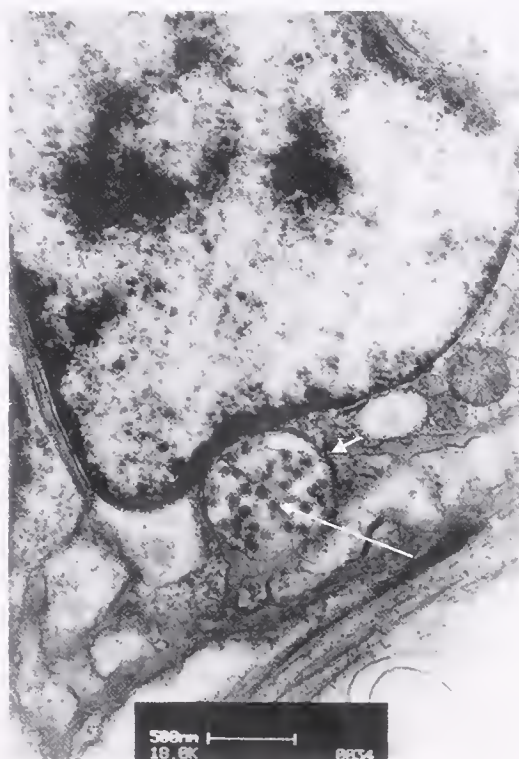


Figure 2. Viral particles (large arrow) in an extension of the nucleus or in a vacuole (small arrow) of the infected cell.

viral particles including capsid shape, presence of a thin wall, size range (Fig. 1), and presence in an extension of the nucleus or in a vacuole of the infected cell (Fig. 2) suggest that they are closely related to Herpes virus (virions enveloped, slightly pleomorphic, spherical, 120–200 nm in diameter). Surface projections of envelope distinct spikes were dispersed evenly over all the surface.

Nucleocapsid surrounded by the tegument that consists of globular material, which is frequently asymmetrically distributed and may be variable in amount. Nucleocapsids were sometimes penetrated by stain (although the intact envelope was impermeable to stain) 100–110 nm in diameter.

Nucleocapsids appeared to be angular and surface capsomer

arrangement obvious. There were 162 capsomers per nucleocapsid (capsomeres hexagonal in cross-section with a hole running half-way down the long axis). The core consisted of a fibrillar spool on which the DNA is wrapped, and the ends of the fibers are anchored to the underside of the capsid shell. Incomplete virus particles often present, are capsids lacking the envelope. Virions contain one molecule of linear double stranded DNA (van Regenmortel et al. 2000). Based on this description of the Herpes viruses, the particles shown in Figures 1 and 2 can be described as herpes-like. There were no particles in the main part of the nucleus and no virions in the cytoplasm. Some capsids were variable in size and thin-walled.

Herpes and herpes-like viruses have been recorded in turtles, carps, ictalurids, frogs, and other aquatic organisms (Buchanan & Richards 1982). In oysters there are several records of herpes or herpes-like viruses around the world (Table 1), and these virus infections in bivalves seem to be ubiquitous (Le Deuff & Renault 1999). All these herpes-like viruses have been described in larval stages and young spat with the exception of adult *Ostrea angasi* in Australia (Hine & Thorne 1997) and possibly adult *Crassostrea virginica* in Maine USA (Farley et al. 1972). However, Farley et al. did not mention the age or size of the infected oysters. Studies on the development of molecular analysis to detect the herpes-like virus in oyster larvae and juveniles have been carried out (Le Deuff & Renault 1999, Renault et al. 2000, Renault & Arzul 2001). Interspecies transmission also has been studied (Arzul et al. 2001a, Arzul et al. 2001b). More recently, Arzul et al. (2002) detected the herpes-like virus in a symptomatic *C. gigas* adult using molecular techniques, including PCR and in situ hybridization (ISH). These authors suggest that this virus may persist in its host after primary infection, and a comparison between adult oysters and infected spat indicated that the spat contain higher amounts of virus DNA. They concluded that viral replication appears less common in adults than in spat. In contrast, our results (Cáceres-Martínez & Vásquez-Yeomans 2003) and those shown in this study, suggest the connection of a herpes-like virus in gill tissue of adults *C. gigas* with clinical signs, histologic alterations, and high mortality episodes. To our knowledge, this is the first record of herpes-like viruses by TEM in adult oysters.

More research is being conducted to further characterize the virus and to determine the influence of environmental factors on the development of the disease in larvae, juveniles, and adults.

TABLE 1.
Herpes-like virus infection in oysters.

Host	Growth Stage	Tissue	Mortality	Name of the Disease	Locality	Reference
<i>Crassostrea virginica</i>	Not mentioned, possibly adult	Haemocytes		Unnamed	Maine, USA	Farley et al. 1972
<i>Crassostrea gigas</i>	Larvae	Connective and mantle epithelium		Unnamed	Northern France	Nicolas et al. 1992
<i>Crassostrea gigas</i>	Larvae	Entire larvae	60–100%	Unnamed	Auckland, New Zealand	Hine et al. 1992
<i>Ostrea edulis</i>	Young spat (5 months old)	Connective, around digestive tubules	90%	Herpes-like infection	Brittany, France	Comps & Cochenne 1993
<i>Crassostrea gigas</i>	Young spat (3–7 months old)	Connective in the gills, mantle and around digestive tubules	80–90%	Unnamed	France, Atlantic Coast	Renault et al. 1994
<i>Ostrea angasi</i>	Adults			Unnamed	Australia	Hine and Thorne 1997

Experimental infections by cohabitation and by injection of a homogenate of eroded gill tissues from symptomatic oysters are being carried out to confirm the role of these herpes virus-like particles as the cause of these mortalities.

ACKNOWLEDGEMENTS

The authors thank anonymous herpesvirus structural expert for his help in the ultrastructural identification of the virus and Dra.

Carmen Paniagua Chávez for suggestions to the manuscript and help in translation. This work was supported by Centro de Investigación Científica y de Educación Superior de Ensenada, México throughout the project number 623106, the project from Consejo Nacional de Ciencia y Tecnología CONACyT 225080-5-3933PB and Instituto de Investigaciones Marinas de Vigo, Spain. R. V.-Y. was supported by a grant of the Consejo Nacional de Ciencia y Tecnología (CONACyT) from México.

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SAND ELIMINATION BY THE ASIATIC HARD CLAM *MERETRIX MERETRIX* (L.): INFLUENCES OF TEMPERATURE, SALINITY AND SEASON

KAREN K. Y. LUI¹ AND KENNETH M. Y. LEUNG^{1,*}

¹The Swire Institute of Marine Science and Department of Ecology & Biodiversity, The University of Hong Kong, Pokfulam, Hong Kong, China

ABSTRACT Clams, being filter-feeders that burrow into sand, tend to accumulate sand in their body tissues. Depuration reduces the clams' gut contents and eliminates sand from different body fractions, providing some cleansing and ensuring that they are safe for human consumption. The Asiatic hard clam *Meretrix meretrix* (Bivalvia: Veneridae) is a commercially important species inhabiting sand flats of Hong Kong and Southeast Asia. In this laboratory study, the effects of temperature and salinity on the rate and profile of sand elimination in *M. meretrix* were studied during winter and summer using a 2 × 2 factorial design (i.e., two temperatures 20°C and 30°C, and two salinities 15‰ and 30‰). Sand removal rate was measured in terms of a decrease in inorganic content (i.e., ash) over a 72-h period. In general, sand was removed from the gut and body tissues and transferred to the external body fluid for subsequent discharge to the ambient water. *Meretrix meretrix* exhibited significantly lower sand removal rates in summer than in winter across all treatments, whereas the highest sand removal rate was observed at 20°C and 15‰ in both seasons. The sand removal rate of *M. meretrix* is, therefore, not only affected by temperature and salinity, but also strongly associated with natural, seasonal, effects. These findings have important implications for optimizing conditions for sand elimination of this species for the shellfish industry in Asia.

KEY WORDS: depuration, salinity, sand elimination, season, temperature

INTRODUCTION

Sandy shore bivalves burrow into sand as an adaptation to protect themselves from wave impact, temperature fluctuations, desiccation, and predation (Nybakken 1997). As filter feeders, they feed during high tide using their siphons, which extend to the sand surface to collect microscopic organisms and particulates from the water column (Morton 1979). Ingested particles, containing both organic and inorganic matter, are either digested in the gut or expelled as faecal pellets (Hylleberg & Gallucci 1975, Ruppert & Barnes 1994). In addition sand particles, together with other unwanted particles such as viruses, bacteria, and pollutants, will be trapped in different organs such as the gills, gut, and adductor muscles (Richards 1988, Savari et al. 1991, Sobsey & Jaykus 1991, Somerset 1991, Ho & Kim 1995, Ariza et al. 1999). When the clams are sold for human consumption, the tissues that contain sand can be unpleasant to eat and also pose a health risk. As a common practice the clams are, therefore, allowed to clean themselves in clean seawater (i.e., depuration) to remove the sand in their body (Richards 1988, Savari et al. 1991).

In bivalves, significant effects of salinity have been reported on mortality (Mane 1978, Morton & Chan 1990), growth (Robert et al. 1988, Hutchinson & Hawkins 1992), and cardiac and siphonal pumping activity (Akberali & Davenport 1981). Salinity is especially important in controlling the effectiveness of depuration by sandy shore bivalves. Richards (1988), for example, demonstrated that salinities between 22‰ and 31‰ were optimal for microbial depuration by the hard clam *Mercenaria mercenaria* (L.), soft-shell clam *Mya arenaria* (L.), and hen clam *Venus gallina* (L.).

Apart from salinity, the depuration rate of bivalves is also influenced by water temperature, suggesting that it may be associated with temperature-dependent metabolic activities. Previous studies have shown that growth (His et al. 1989), respiratory responses (Hicks & McMahon 2002), and filtration rate (Walne 1972) of bivalves are temperature-dependent. Richards (1988) found that the depuration of viruses by the hard clam *Mercenaria*

mercenaria (L.) was improved by increasing temperature, and a similar result was observed in a study on the elimination of coliphages by the mussel *Mytilus edulis* (L.) (Power & Collins 1990). Additionally, Richards (1988) investigated the seasonal variation of microbial depuration by temperate clams in terms of the combined effect of salinity and temperature and revealed that viruses were more effectively depurated from the clams during the summer and fall months than during the winter.

Although the depuration of pathogens (e.g., viruses; Carter & Cantelmo 1990, Ho & Tam 2000), soluble pollutants (e.g., hydrocarbons and heavy metals; Denton & Jones 1981, Jovanovich & Marion 1987, Savari et al. 1991, Reinfelder et al. 1997, Ariza et al. 1999), and phytotoxins (Blanco et al. 2002) in bivalves has widely been studied, information on the elimination rate of insoluble, sand-like particles in clams and cockles is scarce. Indeed, there are only a few reports on sand elimination rates in mussel, clam, and cockle species in relation to their storage and handling in the shellfish industry (De Voys 1987, Richards 1988, NSSP 1990, Somerset 1991).

The current study was therefore designed to investigate the rate of sand elimination by the commercially important, Asiatic hard clam *Meretrix meretrix* (L.) in clean seawater at four different temperature/salinity regimes during winter and summer, respectively. The results from such a study are important for optimizing conditions for sand elimination of this species for the shellfish industry in Asia.

MATERIALS AND METHODS

Sampling and Experimental Design

Meretrix meretrix is one of the largest clam species among the Venus-shells (Veneridae) and very common on sheltered sand flats in Hong Kong, South China, India, Vietnam, Thailand, and the Saudi Arabian Gulf (Morton & Morton 1983, Jayabal & Kalyani 1986, Nhan et al. 1999, Vazquez et al. 1991). As a commercially important clam species in Asia, it has widely been cultured in China and Thailand (Ho & Zheng 1994, Ho & Kim 1995).

In the current study, adult *M. meretrix* were randomly collected

*Corresponding author. E-mail: kmyleung@hkucc.hku.hk

from Shui Hau in Lantau Island, Hong Kong (Grid reference: 22°13'N, 113°55'E) during low tides in winter (24 February 2003; 75 individuals; shell length: 50.01 ± 4.13 mm, mean \pm SD) and in summer (5 June 2003; 75 individuals; shell length: 54.9 ± 7.3 mm). Clams were collected using a specially constructed clam-rack commonly used by fishermen in Hong Kong and South China (Morton & Morton 1983).

Hong Kong has a strongly seasonal, monsoonal climate with a hot, wet summer and cool, dry winter (Kaehler & Williams 1996). Seawater temperature and salinity were measured using a thermometer and a refractometer (ATGAO, S/Mill-E, Tokyo, Japan). The seawater temperature and salinity in the field were 15.0°C and 32.0‰ in winter and 29.0°C and 14.0‰ in summer. Such a considerable difference in salinity between the two seasons was mainly due to the close proximity of the site to the Pearl River estuary, which receives a huge amount of freshwater input during summer (Morton & Wu 1975). The collected clams were transferred to the laboratory within 2 h of collection. On arrival, the clams were divided into four groups and held in 1 L aerated glass tanks filled with 500 mL of artificial seawater (Kent Marine Salt, Kent Marine Inc., Acworth, Georgia) at the same salinity level as measured at the collection site and at room temperature (20°C).

The experimental ranges of temperatures and salinities were based on the natural ranges in the environment (Morton & Wu 1975, Morton & Morton 1983). The clams were acclimatized to four combinations of two temperatures ($20 \pm 0.2^\circ\text{C}$ and $30 \pm 0.4^\circ\text{C}$, mean \pm SD) and two salinities ($15 \pm 0.8\text{‰}$ and $30 \pm 0.4\text{‰}$). The water temperature of the 30°C treatments was adjusted by increasing 4°C every 3 h using a water bath with a temperature controller, whereas the 20°C treatments were maintained at room temperature. Salinity was adjusted by decreasing or increasing values by 5‰ every 3 h until the desired levels were reached. This was achieved by renewing the seawater with artificial sea salts. A control group of 15 individuals was randomly selected at the beginning of the experiment to determine the initial inorganic content of the clams prior to depuration.

For each treatment group, there were three 1-L glass tanks, each holding 5 animals ($\Sigma n = 4 \text{ treatments} \times 3 \text{ tanks} \times 5 \text{ clams} = 60 \text{ clams}$). Water salinity and temperature were kept constant for each treatment and monitored twice daily. Experimental water was renewed daily. No food was provided and a 12-h light:12-h dark cycle was maintained throughout the experiment. In a preliminary study, Lui (2003, unpublished data) observed that *M. meretrix* individuals removed >65% of sand from their body after 72 h in artificial seawater at 20°C and 32‰ (sand remaining [%] = $202.2 \times \exp^{-4.5 \times t} + 34.2$, where t is the duration of sand removal in hours). The clams were, therefore, allowed to depurate over 72 h in the current study, after which all animals were collected for dissection, condition index, and inorganic content measurements.

Dissection of Clams

To study the sand elimination profile and pathway, sand content in three different body compartments, namely the external body fluid, the gut, and the remaining soft body tissues, were determined. The animals in their shells were thoroughly scrubbed with a stiff brush in running tap water, rinsed with distilled water, and left to dry on clean paper towels. Shucking was achieved by inserting a dissecting knife at the site of the adductor muscle. After the shell valves were opened, any liquid was drained and collected in a preweighed aluminum container. This sample was designated

as the external content (EC). The digestive tract was then carefully dissected and designated as the gut content (GC) and placed in a preweighed aluminum container. The remaining body tissues were also placed in another preweighed aluminum container and designated as the body content (BC).

Inorganic Content Analysis and Condition Index

Five empty, preweighed aluminum containers were used as controls for the weighing procedure. All samples, along with the empty shells, were dried in an oven at 80°C for at least 48 h until a constant dry weight was achieved. For each animal, dry weights of all the components (i.e., EC, GC, and BC) and empty shells were weighed using an electronic balance (± 0.00001 g; OHAUS Analytical Plus, Nanikon, Switzerland). Dried samples were then ignited at 500°C in a muffle furnace for 4 h (Takada 1995) and the remaining ash content weighed. Three readings were taken for each sample to ensure a precise mean value for representing the dry ash weight. The inorganic content of each component was calculated as the percentage of the ash weight of the total dry weight of the whole soft-body tissues. Total inorganic content percentage (TIC, %) of each clam was calculated using the following equation:

$$\text{TIC (\%)} = (\text{Total Ash Weight/Total Dry Soft-Body Tissue Weight}) \times 100\% \quad (1)$$

where total ash weight was calculated by summation of the ash weights from all of the three fractions (EC, GC, and BC) and total dry tissue weight was the sum of the dry weights of the gut and body tissues.

The condition index (CI) was calculated using the following equation described in Beninger and Lucas (1984) and expressed as g tissue per g shell weight:

$$\text{CI} = \text{Dry Soft-Body Tissue Weight/Dry Shell Weight} \quad (2)$$

The Rate of Sand Elimination

The rate of sand elimination ($\text{mg g}^{-1} \text{ h}^{-1}$) for each species was calculated using the following equation:

$$\text{Rate} = (\text{TIC}_{\text{final}} - \text{TIC}_{\text{initial}})/\text{Depuration Time} \quad (3)$$

where $\text{TIC}_{\text{final}}$ is the total inorganic concentration (mg g^{-1} dry soft-body weight) at the end of the experiment over the total depuration time, and $\text{TIC}_{\text{initial}}$ is the initial total inorganic concentration (mg g^{-1} dry soft-body weight) at time zero. The results were also expressed as percent sand remaining, which was a ratio of sand content between the control clams (i.e., initial) and each treatment group after 72 h depuration.

Statistical Analysis

Data were tested for homogeneity of variances using Levene's test (SPSS version 10.0). Two-way analysis of variance (ANOVA) was used to test the effect of the treatment (five levels: control and four temperature/salinity regimes) and season (two levels: winter and summer) on the condition index. To compare the inorganic content in each individual components (i.e., EC, GC, and BC) among the control and the four regimes in each season, one-way ANOVAs were used followed by *post hoc* Tukey's multiple comparison tests to identify significantly different means. Finally, a three-way ANOVA was used to test the significance of the effects of temperature (two levels), salinity (two levels), and season (two levels) on the sand removal rate.

RESULTS

Condition Index

As no food was provided during the depuration period, the condition index (CI) of *M. meretrix* decreased significantly after the 72 h depuration for the four treatments in both seasons (Fig. 1; two-way ANOVA: treatment effect, $F_{4, 10} = 35.64$, $P < 0.001$). The CI values were significantly lower in summer than in winter across all of the control and treatment groups (Fig. 1; two-way ANOVA: season effect, $F_{1, 19} = 34.74$, $P < 0.001$).

Inorganic Content in Different Body Compartments

Before depuration, the distribution of sand in the control group of *M. meretrix* followed the order external body fluid (EC) > body tissues (BC) > guts (GC) during the summer (Figs. 2a, 2c, and 2e). In contrast, higher levels of sand grit were found in BC than EC in the control *M. meretrix* group during the winter (Figs. 2b, 2d, and 2f). After the 72-h depuration period, the inorganic content in the gut decreased across all treatments in both seasons, although this decline in the clams exposed to 30‰ and 20°C was not statistically significant (Figs. 2c and 2d). For treatments under high salinity (30‰) regardless of their water temperature and season, the amount of sand depurated was small, and the EC accounted for the largest proportion of overall inorganic content (Figs. 2a and 2b). *Meretrix meretrix* collected in winter and exposed to the two high-salinity regimes showed a significant increase in inorganic content in the EC over the depuration period (Fig. 2b). In terms of total inorganic contents (TIC) per individual clam (Figs. 2g and 2h), the two low-salinity (15‰) treatment groups exhibited significant reductions in their sand contents, whereas the two high-salinity (30‰) treatment groups did not show any significant decline in TIC. These results indicate that the speed of transportation of sand from the body tissues and gut to the external fluid for discharge was much slower in *M. meretrix* exposed to high salinities (30‰) than for the other treatment groups at low salinity (15‰).

The sand removal rate of *M. meretrix* was strongly affected by the different temperature/salinity regimes and season (Figs. 3a and 3b). In general, significantly higher sand removal rates (or lower sand remaining %) were observed at low salinity and low temperature (Fig. 3; Table 1), with the highest elimination rate at 15‰

and 20°C for both seasons (Fig. 3). The rate of sand removal was, however, significantly lower in the summer than in the winter (Fig. 3; Table 1). When comparing the magnitude of the differences among the four groups and the F values (Fig. 3b; Table 1), it was clear that salinity was a more important factor affecting sand elimination rates in *M. meretrix* than temperature and season. This was further supported by the absence of any significant interactions between temperature, salinity, and season on the rate of sand removal (Table 1).

DISCUSSION

Sand Distribution and Depuration

In this study, sand (as determined by inorganic content) was transported and accumulated in different body compartments. Sand particles either passed through the gut and were discharged as feces or pseudofeces or were transported from the tissues to the external body fluid for subsequent disposal from the edge of the shell. During a 72-h depuration period, a considerable amount of sand particles was defecated from the gut by *M. meretrix* in both seasons. The inorganic content remaining in the gut might serve several adaptive functions. First, inorganic matter might contain some essential nutrients that are assimilated through the gut or may have microalgal food items bound to their surface (Huz et al. 2002). Second, Chia (1973) demonstrated that sand grains could be used as a weight belt for juvenile sand dollars, *Dendraster excentricus* (Eschscholtz), that stored these grains in their intestinal diverticulum to help them settle on the sea floor. There is, however, much less information available on the adaptive function of sediment accumulation in burrowing clams.

Salinity Effect

Sand removal rate in *M. meretrix* was higher at low salinities (15‰). A similar result has been reported for the blue mussel, *Mytilus edulis*, and the hard clam, *Mercenaria mercenaria*, concerning the depuration of coliphages and *Escherichia coli* (Richards 1988, Power & Collins 1990). Such an increase in depuration rate may be attributable to an increase in filtration rate and pumping activity at low salinities. Nonetheless, Richards (1988) also reported that other species, such as the soft-shell clam *Mya arenaria* (L.), showed a greater depuration rate at higher salinities (25–30‰), suggesting that the effect of salinity is species-specific. Sobsey and Jaykus (1991) also reported that the range of optimal salinity for effective depuration varies among different bivalve species.

Variation in salinity affects bivalve physiology in a number of ways. Hutchinson and Hawkins (1992) demonstrated that the excretion rate of the oyster *Ostrea edulis* (L.) increased at low salinities, whereas the effect of salinity on their growth varied, depending on the level of food availability. Shell valve activity in response to variation in salinity may be another factor controlling the rate of sand elimination. Akherali and Davenport (1981), for example, showed that the exhalant siphon and valves of the clam *Scrobicularia plana* (de Costa) closed at low salinities and thus prevented their pumping activity.

In the current study, *M. meretrix* removed more sand at low salinities (15‰), suggesting that they perform well under lower salinities. *Meretrix meretrix* occurs on shores in Hong Kong where surface salinities may fall to as little as 2‰ as a result of the summer monsoons and the influx of freshwater from the Pearl

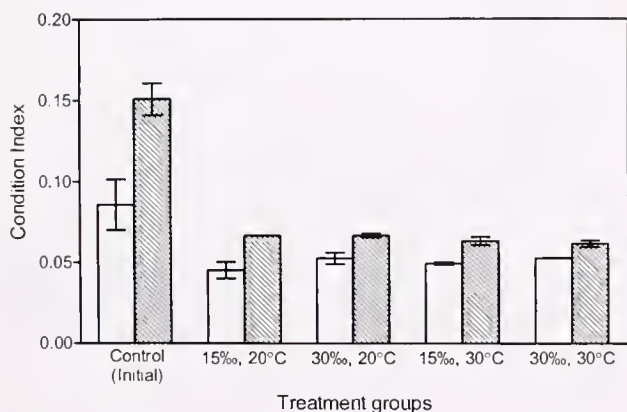


Figure 1. Condition index for *Meretrix meretrix* (mean ± SEM) before and after depuration in artificial seawater at different salinity/temperature regimes for 72 h during summer (open bars) and winter (shaded bars).

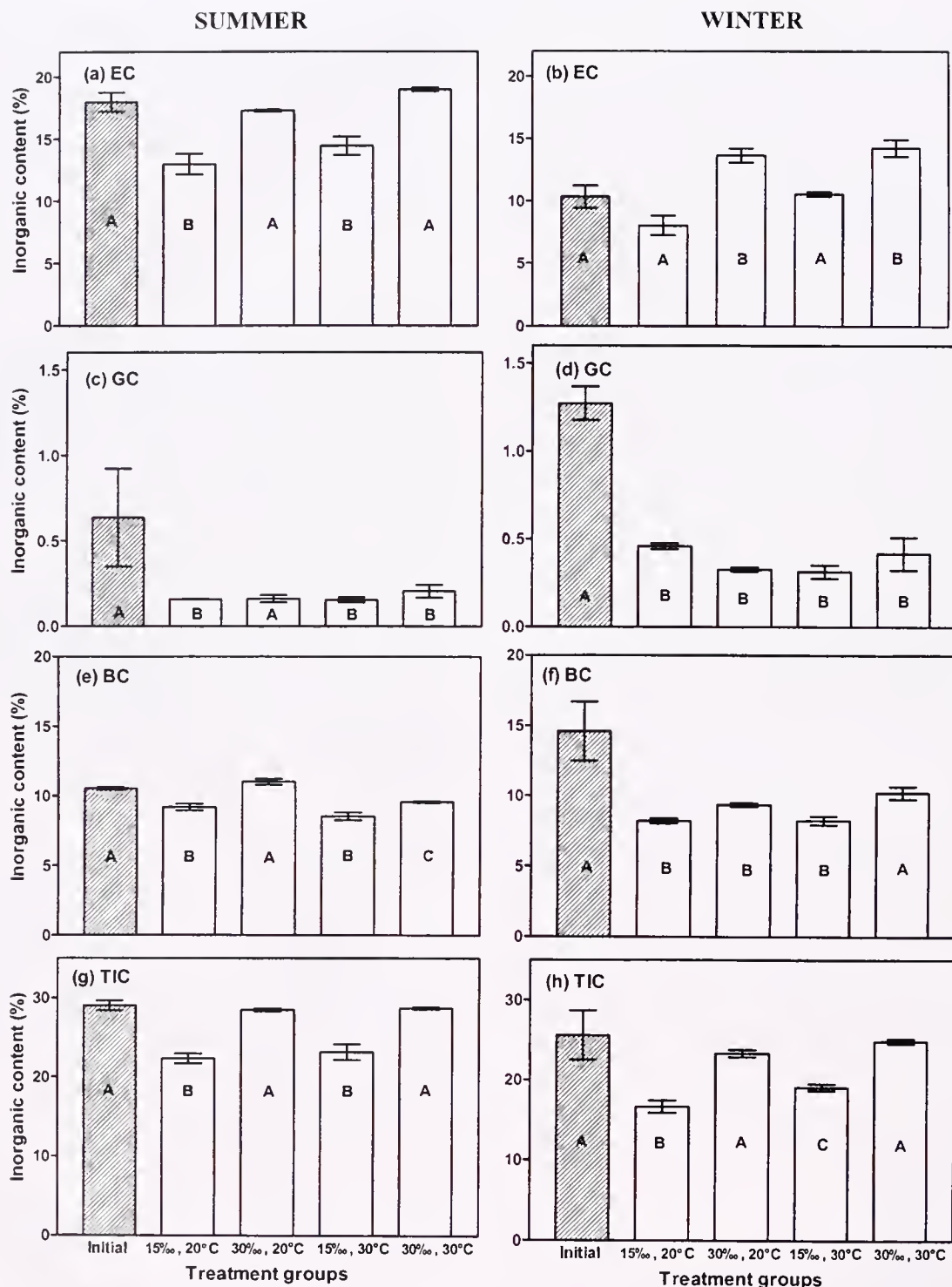


Figure 2. Mean (\pm SEM) inorganic contents in (a, b) external body fluid, (c, d) gut, (e, f) body tissues, and (g, h) whole individuals of *Meretrix meretrix* at different salinity/temperature regimes after 72-h depuration period during summer (left column) and winter (right column). Initial values before depuration are shown as shaded bars. Scales for the y-axis vary among the eight figures. In each figure, bars with the same letter are not significantly different (Tukey's multiple comparison test, $P < 0.05$).

River (Morton & Morton 1983). This ability to survive in low salinities is important for infaunal species, as the salinity of estuarine and intertidal zones can fluctuate on an hourly, daily, weekly, and seasonal basis (Gainey & Greenberg 1977, Leung et al. 2002). This may explain why the effect of salinity on sand elimination in *M. meretrix* did not vary between winter and summer.

Temperature Effect

Sand elimination by *M. meretrix* is also temperature-dependent; higher removal rates of sand were generally found at lower temperatures (20°C). Some shellfish sellers in Hong Kong use lower water temperatures to achieve effective sand depuration by clams.

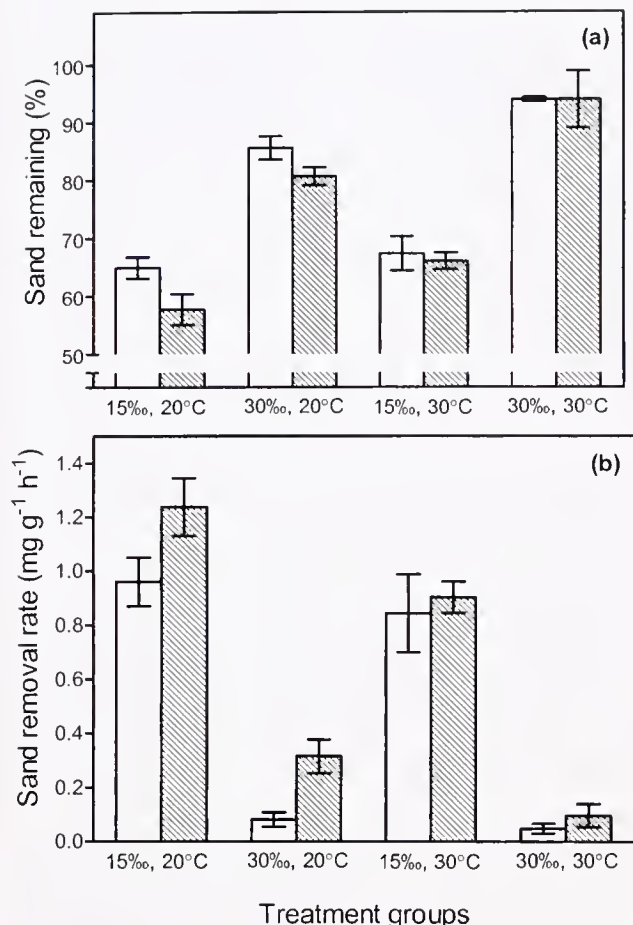


Figure 3. The percentage of (a) sand remaining and (b) depuration rate of *Meretrix meretrix* exposed to different salinity/temperature regimes during summer (open bars) and winter (shaded bars). Mean and SEM are presented.

as survival rates of the shellfish are generally higher at lower temperatures such as 17–18°C (Lui, personal communication). Matthews and McMahon (1999) also demonstrated that the zebra mussel, *Dreissena polymorpha* (Pallas), and the Asian clam, *Corbicula fluminea* (Muller), showed a higher tolerance to hypoxia at lower temperatures. In contrast, however, the West African clam, *Galatea paradoxa* (Born), showed a greater removal of sand at elevated temperatures (Ekanem 2000), whereas the depuration of *E. coli* also increased at higher temperatures in *Mytilus edulis* (Power & Collins 1990) and the king scallop, *Pecten maximus*, (L.) (Heath & Pyke 2001), suggesting that such temperature-dependent responses also vary between species.

Generally, in bivalves, higher water temperatures result in increased feeding rates (in terms of filtration rates; Walne 1972) and higher metabolic rates (in aspects of growth; Robert et al. 1988) and oxygen consumption (Hicks & McMahon 2002). Some workers, however, did not find any significant effects of temperature at the organism level (Denton & Jones 1981, De Vooy 1987, Inza et al. 1998, Blanco et al. 2002), and they argue that the depuration process is independent of metabolic rates, as their target species still efficiently depurated over a broad range of temperatures (6.6–20.5°C). Whether depuration is a temperature-dependent physiologic process in all bivalves species has, therefore, yet to be confirmed.

Combined Effects of Salinity, Temperature, and Season

The effect of temperature on sand elimination by *Meretrix meretrix* was not as pronounced as the effect of salinity. *Meretrix meretrix* must tolerate a wide range of salinities on western shores of Hong Kong. The effect of temperature shock (either hot or cold) is, however, likely to be minimal on the shore because they burrow into the sediment where temperatures are more stable as compared with air temperatures (Yang et al. 2003).

In *M. meretrix*, optimal sand elimination was achieved at low temperatures (20°C) and salinities (15‰) in both summer and winter. Although *M. meretrix* exhibited a higher rate of sand removal in winter than summer, the influence of temperature and salinity was similar between the two seasons. Interestingly, the significantly lower condition indices for *M. meretrix* in the summer (June) were probably associated with low food (i.e., particulate organic matter) availability in the water column (Wong & Cheung 2003), which may possibly be due to heat stress and the increase in freshwater input from rainfall and surface runoff. In contrast, micro- and macroalgae are more abundant during winter in Hong Kong coastal environments (Morton & Morton 1983, Hodgkiss 1984, Nagarkar & Williams 1999) and may considerably enhance the growth of the clams. In such favorable conditions in winter, the clams not only have better physiologic status to efficiently ingest and assimilate nutrients but are also able to excrete waste and depurate sand more effectively. Jovanovich and Marion (1987) also observed a seasonal variation in the depuration of anthracene by the clam *Rangia cuneata* (Sowerby); depuration rates remained low from March to August and rapidly increased to the highest levels during the fall spawning period. This seasonality was related to the reproductive cycle of *R. cuneata* and associated biochemical changes, which in turn affected the physiologic state of the clam.

The oyster *Ostrea edulis* (L.) shows physiologic variation according to seasonality (Hutchinson & Hawkins 1992). A “winter” physiologic state enables *O. edulis* to survive at low temperatures and salinities that are normally encountered during the winter months in its shallow coastal water habitat, whereas a reverse physiologic state was recorded in summer. Similarly, siphonal activity of the soft-shell clam, *Mya arenaria* (L.), was higher during May–September and then strongly declined from October (Thorin 2000). This variation was mainly attributed to seasonal changes in resource availability, water temperature, or individual reproductive state of *M. arenaria*. To our knowledge, the natural reproductive cycle of *M. meretrix* has yet to be established in Hong Kong and South China, although they have been artificially reared or cultured in Southeast Asia. As a result, we are unable to comment on the possible influence of their reproductive cycle on sand elimination.

Conclusions

Sand can be trapped in different body compartments in burrowing bivalves, and this needs to be eliminated for the clam to cleanse itself in the natural environment and for use in the shellfish industry to ensure safety for human consumption. Simple methods for sand elimination using clean seawater or freshwater are currently used by shellfish sellers and seafood restaurant owners worldwide (De Vooy 1987, Carter & Cantelmo 1990, NSSP 1990, Ho & Tam 2000, Heath & Pyke 2001). This pilot study investigated the effect of salinity, temperature, and season on sand elimination in the commercially important clam *Meretrix meretrix*. The rate of sand removal by *M. meretrix* was significantly affected by

TABLE 1.

Results of three-way ANOVA on the sand depuration rate ($\text{mg g}^{-1} \text{h}^{-1}$) of *Meretrix meretrix* exposed to the four different salinity and temperature regimes during the summer and winter.

Factor	df	MS	F	P Value
Temp.	1	0.174000	8.625	0.010*
Salinity	1	4.087000	202.487	<0.0001***
Season	1	0.135000	6.683	0.021*
Temp. \times Salinity	1	0.014140	0.701	0.416
Temp. \times Season	1	0.057400	2.844	0.112
Salinity \times Season	1	0.001029	0.051	0.824
Temp. \times Salinity \times Season	1	0.000383	0.019	0.892
Residual	15	0.020180		

Asterisks denote significant factors at * $P < 0.05$; *** $P < 0.001$.

salinity and temperature. The highest rate of sand removal was found at low salinities (15‰) and temperatures (20°C). These optimal depuration conditions were identical in both summer and winter, suggesting that lower temperature and lower salinity were physiologically more favorable regardless of seasonality. Depuration rates in the clams were, however, higher in winter for all treatment groups, and this is probably associated with seasonal variation in the climate, food availability, and reproductive cycle of the clam. Further investigation is, however, required to test whether factors other than salinity and temperature, such as repro-

ductive cycle and food availability, could be important in regulating the rate of sand depuration in *M. meretrix*.

ACKNOWLEDGMENTS

The authors thank Cecily Law, Laura Wong, and Lily Ng for their technical support and Cyrus Cheng and Shirley Lui for helping with collecting the samples. Special thanks are also extended to Gray A. Williams, Will Trehwella, Valerie Ho, Justine Tsui, Danny Lau, and Jasmine Ng for their critical reading of earlier drafts of this manuscript.

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SURFCLAM HISTOPATHOLOGY SURVEY ALONG THE DELMARVA MORTALITY LINE

YUNGKUL KIM AND ERIC N. POWELL

Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, New Jersey 08349

ABSTRACT The 2002 National Marine Fisheries Service–Northeast Fisheries Science Center (NMFS-NEFSC) survey revealed a substantial reduction in the abundance of surf clams, *Spisula solidissima*, off the Delmarva Peninsula. Increased mortality since 1999 was focused inshore of a southeast trending line extending from approximately the Delaware Bay mouth to the central continental shelf off the mouth of Chesapeake Bay. To investigate further the proximate causes of mortality, we carried out a histopathological survey of surf clams collected along this southeast-trending “mortality” line. The most northern stations had the highest condition indices, the lowest gonadal abnormality scores, with one exception, and the lowest digestive gland atrophy scores. Animals at the stations with lowest average condition index had body weights less than 65% of the average animal taken at the station with the highest condition index. A syndrome describing a malnourished state common in clams taken along the mortality line emerged from this analysis. Animals with low condition were also animals with higher scores of gonadal abnormality and higher scores of digestive gland atrophy. Neither parasites, nematodes and cestodes, occurred commonly, nor instances of hemocyte infiltration, also common, were significantly correlated with low condition index and the associated tissue disorders indicative of malnutrition. Nematodes and the frequently associated instances of focal infiltration by hemocytes were not obviously more common in the southern stations where condition index was low. Cestodes tended to occur more frequently at sites where condition index was low, but infection intensity was high at some stations not so characterized. No disease-causing organisms were observed in this study. Starvation, brought on by regional shifts in temperature mismatching food supply and feeding rate with tissue maintenance needs, offers one potential mechanism explaining the distribution of apparently malnourished clams and the origin of the increase in mortality inshore of the “mortality” line.

KEY WORDS: surfclam, *Spisula*, histopathology

INTRODUCTION

Surf clams, *Spisula solidissima*, support one of the largest fisheries in the Mid-Atlantic Bight and represent a biomass dominant for much of the inner half of the continental shelf of that region (Merrill & Ropes 1969, Weinberg 1998, Weinberg 1999, NEFSC 2003). Prior to 1999, surf clams were abundant from northern Virginia to inshore Long Island, with discontinuous but significant abundances along the southern New England coast and onto Georges bank (Theroux & Wigley 1983, NEFSC 2003). In 1999, estimated stock biomass, just in the Exclusive Economic Zone (EEZ), exceeded 1 million MT (NEFSC 2003), a stock biomass estimated to be at or near carrying capacity throughout much of the surf clam’s range. Surf clams are long-lived, exceeding 30 y in the oldest specimens (Weinberg 1999). Natural mortality rate is estimated to be 0.15 y^{-1} and current fishing mortality rate is less than half this value (NEFSC 2003). Changes in adult abundance thus occur slowly as a consequence of normal population dynamics.

The 2002 National Marine Fisheries Service–Northeast Fisheries Science Center (NMFS-NEFSC) survey revealed a substantial reduction in surf clam abundance off the Delmarva Peninsula (NEFSC 2003). Weinberg (1998) had earlier documented that clams in this region were slower growing than clams from more northern climes. NEFSC (2003) documented that the increased mortality since 1999 was focused inshore of a southeast trending line extending from approximately the Delaware Bay mouth to the central continental shelf off the mouth of Chesapeake Bay. Figure 1 shows the NMFS-NEFSC catches of surf clams during the 2002 survey in this region. No living surf clams were caught inshore of this mortality line, in contrast to the pre-1999 period when surf clams were caught in abundance over much of this area. Since 1999, yearly mortality rate has been well above the stock-wide average of 0.15 y^{-1} previously estimated for this region. The southeastern trend, which follows more or less the isothermic structure of the region, supports the assumption that an underlying causal influence is rising sea temperatures. Weinberg et al. (2002) suggested that increasing temperatures in the Mid-Atlantic Bight

associated with global temperature rise might significantly influence surfclam growth and mortality.

A recent survey of inshore Maryland waters by Powell (2003) reoccupying stations originally sampled by Loesch and Ropes (1977) found few living surf clams inshore of the EEZ along the same stretch of coastline. Although the time when surf clams disappeared from this inshore region is not well-documented, the inshore survey expanded the zone south of Delaware Bay where surf clams, once-abundant, are no longer significant contributors to community biomass. The 2003 inshore New Jersey survey carried out by the New Jersey Department of Environmental Protection documents a substantial decline in the abundance of surf clams inshore of the EEZ off New Jersey as well (NJDEP, personal communication), suggesting that a widespread range contraction along the southern and inshore range boundary of the surf clam may be underway. This boundary probably is determined by temperature, as surf clams do not survive well over the summer in more southern climes (Spruck et al. 1995, O’Beirn et al. 1997) and summer temperature determines faunal boundaries of many species within the Virginian biogeographic province (Cerame-Vivas & Gray 1966).

Surf clams enter high-temperature stress when temperatures exceed 23°C or thereabouts (Loosanoff & Davis 1963, Cable & Landers 1974, Goldberg 1989, Clotteau & Dubé 1993, Walker et al. 1997). Weinberg et al. (2002) observed that bottom water temperatures in the offshore Delmarva region were unlikely to exceed these levels. Thus, a direct effect of temperature as an agent of mortality seems unlikely. However, small increments in temperature above optimal can significantly impact scope for growth in bivalves, a phenomenon often made most manifest in the larger adult animals (Taylor 1960, Hofmann et al. 1994), and temperature-dependent diseases and pathologies are well-known (e.g., Miller & Lawrenz-Miller 1993, Powell et al. 1996, Cook et al. 1998). Either could mediate the influence of temperature as it ultimately modulates the rate of natural mortality. To investigate further the proximate causes of mortality, we carried out a histopathological survey of surf clams collected along the southeast-trending “mortality” line.

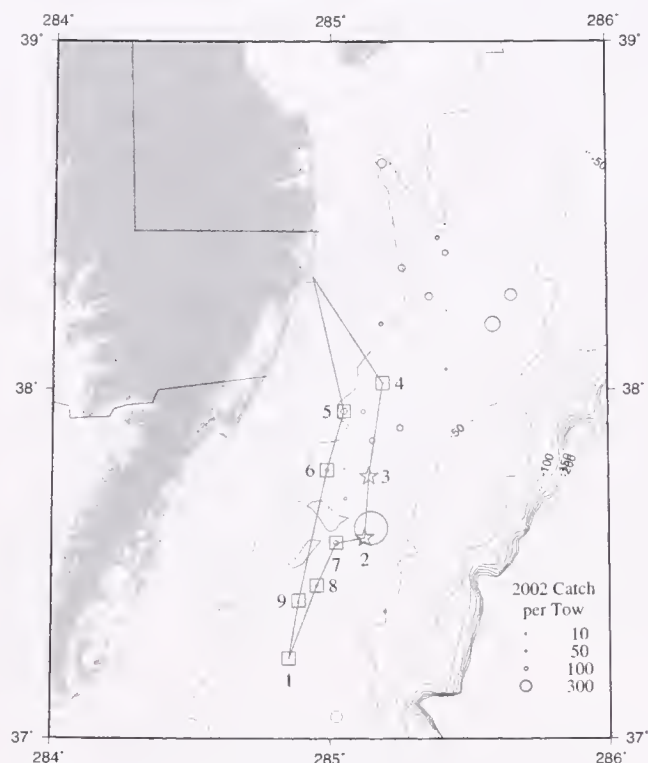


Figure 1. August 28, 2003, cruise track of the *F/V Betty C.* Circles of diameter proportional to catch number (see scale) document catches by the NEFSC survey vessel *R/V Delaware II* in 2002 (NEFSC 2002). Stations where no surf clams were caught in the 2002 survey, mostly inshore of the region identified by positive catches in this figure, are not shown. The mortality line runs just west (inshore) of the line established by stations 1, 5, 6, and 9. Squares indicate the seven selected 2002-survey stations reoccupied by the *Betty C.* Two additional stations chosen based on captains' reports are indicated by a ★. Numbers indicate the station numbers assigned to each station for analysis.

METHODS

Sample Collection and Preparation

Samples were collected by the *F/V Betty C.*, homeport Ocean City, Maryland, on August 28, 2003. The cruise track ran the

mortality line from offshore of the Maryland/Virginia border to about the latitude of the mouth of Chesapeake Bay (Fig. 1). A selection of seven of the inshore-most stations at which the 2002 NEFSC survey found living surf clams was resampled. An additional two stations (stations 2 and 3, Figure 1) were sampled based on captains' reports of "sickly-looking" surf clams in commercial catches.

Samples were taken by hydraulic dredge (Wallace & Hoff, in press). Between 10 and 15 animals, catch being sufficient, were selected from each dredge haul for histopathological analysis (Table 1). In all, 105 surf clams were analyzed. The maximum anterior-posterior shell length of each animal was measured. Measured animals were then opened immediately using a stainless steel knife by cutting the adductor muscles. The shucked clam meats were placed in a bucket of seawater, rinsed to remove sand and other hard particles forced into the mantle cavity during capture, drained, and weighed. Immediately after weighing, a 2-cm-thick dorsal-ventral cross-section of tissue including visceral mass, mantle, gill and foot, was removed from each clam using a scalpel. Each section was stored in a glass jar filled with Davidson's fixative for two days until replaced by 70% ethanol for storage (Ellis et al. 1998a).

Tissue Preparation

Methods followed NOAA Status and Trends protocols (Ellis et al. 1998a, Ellis et al. 1998b) except that tissue subsections were excised prior to embedding due to the large size of the clam cross-sections, rather than embedding an entire cross-section. Target tissues included mantle, gill, kidney, gonad, digestive gland, and connective tissue. Tissue samples were embedded in paraffin after dehydration and clearing. The paraffin-embedded tissue blocks were first sliced at 20 μm to expose an entire tissue section. The tissue-paraffin block was then placed in a freezer overnight before final sectioning at 5 μm . Tissue sections were deparaffinized and hydrated using a xylene-ethanol series, stained in a pentachrome series, dehydrated in a series of acetic acid dips followed by acetone, cleared in xylene, and mounted in Permount (Ellis et al. 1998b).

Histopathological Analysis

Tissue sections were examined under the microscope using a 10 \times ocular and a 10 \times objective. When necessary, a 25 \times or 40 \times

TABLE 1.

Average surf clam length, wet meat weight, and condition index, modal gametogenic stage, and the number of females and males analyzed from each station.*

Station	Weight (g)	Length (mm)	Condition (g mm ⁻¹)	Modal Gametogenic Stage	Number Female	Number Male
1	112.8	138.1	0.81	S3,5	7	6
2	77.6	133.1	0.58	S4,5	10	5
3	91.7	137.9	0.66	S3	10	5
4	133.9	154.9	0.86	D4	4	6
5	125.6	143.4	0.87	5	3	7
6	135.0	148.5	0.91	S4,5	5	5
7	103.1	141.7	0.72	D4	6	9
8	80.1	134.2	0.60	S3,D4	6	9
9	69.0	117.0	0.55	S3,S4	2	0

* Modal gametogenic stage is the most common gametogenic stage at the station.

objective was used for closer examination. All parasites and pathologies were scored for intensity based on either a quantitative or semi-quantitative scale. Quantitative scores were used for parasites that could be tallied individually, including prokaryotic inclusion bodies, nematodes, and cestodes. Each nematode cross-section observed was counted, although a single individual may be responsible for a number of tissue cross-sections. Certain tissue pathologies were also quantified by direct counts, including intense localized (focal) or diffuse infiltration of hemocytes.

Some conditions were assigned to semiquantitative scales depending on the intensity or extensiveness of the affected area. These include so-called digestive gland atrophy, characterized by thinning of the digestive tubule epithelium, and abnormal gonadal development, characterized either by unusual development of gametes at the base of the follicles, by an elevated presence of foreign cells and cellular debris in the follicles, or by immature gametes floating free within the follicular lumen. The semiquantitative scales used are defined in Tables 2 and 3.

Discretely counted parasites and pathologies, such as focal and diffuse infiltration of hemocytes, nematodes, and cestodes, were described in terms of their prevalence and infection intensity. Prevalence, the fraction of individuals with the parasite or pathology, was calculated as:

$$\frac{\text{Number clams affected}}{\text{Number clams analyzed}}$$

Infection intensity, the average number of occurrences of a parasite or pathology in the infected individuals only, was calculated as:

$$\frac{\sum_{i=1}^n \text{Number of occurrences of parasite or pathology}}{\text{Number of affected hosts}}$$

Certain statistical procedures, such as principal components analysis (PCA), necessitated combining uninfected and infected individuals by giving the uninfected individuals an infection intensity of 0. The average value, termed weighted prevalence or mean abundance, was calculated as

$$\text{Prevalence} \times \text{infection intensity.}$$

Tissue disorders measured using semiquantitative scales, such

TABLE 2.

Semiquantitative scale used for the evaluation of digestive gland atrophy, adapted from Ellis et al. (1998b).

Score	Description
0	Normal epithelial thickness in most tubules (0% atrophy), lumen nearly occluded, few tubules even slightly atrophied.
1	Average epithelial thickness less than normal, but greater than one-half normal thickness; most tubules showing some atrophy, some tubules still normal.
2	Epithelial thickness averaging about one-half as thick as normal.
3	Epithelial thickness less than one-half of normal; most tubule epithelia significantly atrophied, some epithelia extremely thin (fully atrophied).
4	Epithelium extremely thin (100% atrophied); nearly all tubules affected.

TABLE 3.

Semiquantitative scale used for the evaluation of abnormal gonadal development.

Score	Description
0	Normal gonad.
1	Less than half the follicles are affected.
2	About half the follicles are affected.
3	More than half the follicles are affected.
4	All follicles affected.

as digestive gland atrophy and abnormal gonadal development, were described in terms of the arithmetic mean of their semiquantitative-scale values.

Miscellaneous Analyses

Condition index was calculated as:

$$\frac{\text{Wet meat weight (g)}}{\text{length (mm)}}$$

Gametogenic stage was assigned based on the stages set forth for mussels by Ellis et al. (1998a) (Table 4). These stages can be condensed into the simpler groupings of Ropes (1968). For some statistical analyses, the gametogenic stages were compressed into three larger categories: developing gonad (all D stages, Table 4), fully developed or ripe gonad (stage 5, Table 4), and spawning and partially spent gonad (all S stages, Table 4).

RESULTS

Condition

Condition index was lowest at stations 2, 3, 8, and 9 (Table 1). Average condition index was at or below 0.60 g mm^{-1} for three of these stations, about 34% lower than the station with the highest average, station 6. The most northern group of stations and the most southern station, station 1, had the highest condition indices, averaging above 0.80 g mm^{-1} .

Most surf clams at the northern stations were in a late stage of gonadal development or ready to spawn (Tables 1 and 5). Most surf clams at the southern stations had begun spawning, but few animals had completed spawning. Spent animals were rare (Table 5). These observations generally agree with Ropes (1968) (see also Jones 1981, Chintala & Grassle 1995).

Parasites

Two parasites were common, nematodes and cestodes. Larval cestodes were found in nearly all tissues. Cestodes were reported in *Spisula similis* by Cake (1977), but appear to be undocumented previously in *S. solidissima*. Most cestodes were observed in either the digestive gland, particularly the connective tissue around the gut or attached to the gut epithelium (Plate 1), or in the gonads. A complete accounting of observations by tissue type is: digestive gland (201 observations), gonad (61 observations), mantle (15), kidney (10), blood vessels (10), foot (6), muscle tissue (4), and gill (1). The formation of a thick tissue capsule surrounding the cestode was a frequently observed host reaction. Encapsulated cestodes appeared to be disintegrating and in the process of resorption.

Cestode prevalence fell below 50% only at station 2. Surf clams

TABLE 4.

Stages used to describe the surf clam gametogenic cycle, adapted from Ellis et al. (1998a).

Stage	Description
Resting/spent gonad	
Stage 0	Inactive or undifferentiated.
Developing gonad	
Stage D1	Gametogenesis has begun; no ripe gametes visible.
Stage D2	Ripe gametes present; gonad developed to about one-third of its final size.
Stage D3	Gonad increased in mass to about half the fully ripe condition; each follicle contains, in area, about equal proportions of ripe and developing gametes.
Stage D4	Gametogenesis still progressing; follicles contain mainly ripe gametes.
Ripe gonad	
Stage 5	Gonad fully ripe, early stages of gametogenesis rare; follicles distended with ripe gametes; ova compacted into polygonal configurations; sperm with visible tails.
Spawning gonad	
Stage S4	Active emission has begun; sperm density reduced; ova rounded off as pressure within follicles is reduced.
Stage S3	Gonad about half empty.
Stage S2	Gonadal area reduced; follicles about one-third full of ripe gametes.
Stage S1	Only residual gametes remain; some may be undergoing cytolysis.

infected by cestodes were most common at stations 1, 7, and 9, where prevalence reached or exceeded 85% (Table 6). These are among the most southern stations. Cestode infection intensity was highest at stations 6, 8, and 9. Infection intensity exceeded 6 observations per clam subsection in surf clams taken from these stations (Table 6).

Larval nematodes were observed in nearly all tissues of surf clams in this study: gonad (77 observations, Plate 2), digestive gland (23 observations), the visceral mass between the body wall and the underlying muscle layer (17), foot (8), muscle tissue (2), and gill (1). In some cases, no conspicuous host response was observed. Frequently, however, hemocyte infiltration was observed in association with the worm. Nematodes were found in surf clams collected from all stations (Table 6). Prevalence was highest, exceeding 50%, at stations 1, 7, and 8. Two of these stations are among the three stations in which cestode prevalence was highest. The high prevalence of nematodes in surf clams off the Delmarva Peninsula is anticipated from previous reports. Nematodes were observed to frequently parasitize surf clams by Lichtenfels et al. (1976, 1978) and Perkins et al. (1975). Nematode infection intensity was highest, reaching at least 3 observations per clam subsection, at stations 1, 4, and 5, but exceeded 2 observations per clam subsection at all stations (Table 6). The distribution of nematode infection intensity diverged markedly from that of cestode infection intensity.

A variety of other parasites were found sporadically, including prokaryotic inclusions, often termed rickettsial or chlamydial bodies (Ellis et al. 1998b), also reported to infect surf clams by Otto

et al. (1979), an unidentified worm, and the haplosporidian hyperparasite of larval nematodes, previously reported by Lichtenfels et al. (1978), Payne et al. (1980), Perkins (1979), and Perkins et al. (1975). Prevalence of prokaryotic inclusions was 16%, nearly all cases being located in the digestive tract epithelium. Haplosporidians were observed rarely, hyperparasitizing nematodes in only 4% of the surf clams harboring nematode parasites. One surf clam was observed to have two unidentified worms, one in the mantle and the other in the foot, similar to the echinostomes observed in the gonoducts of Gulf of Mexico oysters by Ellis et al. (1998b) and later identified by Winstead et al. (1998).

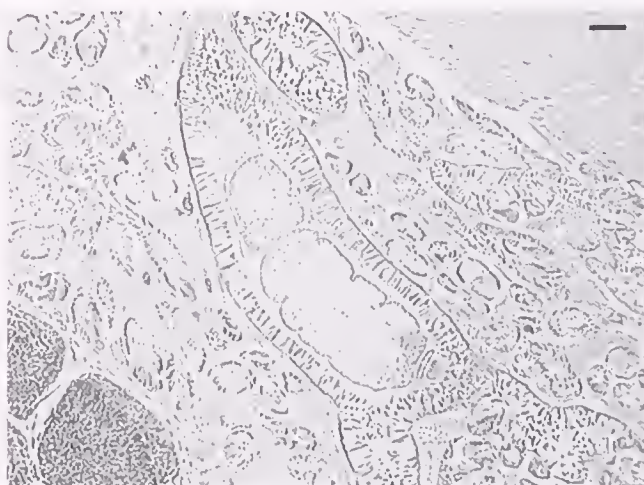
Pathologies and Related Conditions

Despite the nomenclatural connotations, neither digestive gland atrophy nor the disorders referred to as abnormal gonadal development are necessarily pathologic. Digestive gland atrophy manifests itself as a thinning of the digestive tubule cells (Ellis et al. 1998b; Table 2). The condition appears to be representative of poor nutrition in some bivalves (e.g., Palmer 1979, Winstead

TABLE 5.

Frequency of observation of each gametogenic stage defined in Table 4.

	Gametogenic Stage								
	D1	D2	D3	D4	5	S4	S3	S2	S1
Number of observations	1	0	3	25	25	15	29	6	1
Instances of abnormality	1	0	3	12	8	6	19	5	1

Plate 1. Cestode in the digestive tract lumen of a surf clam. Scale bar is 100 μ m.

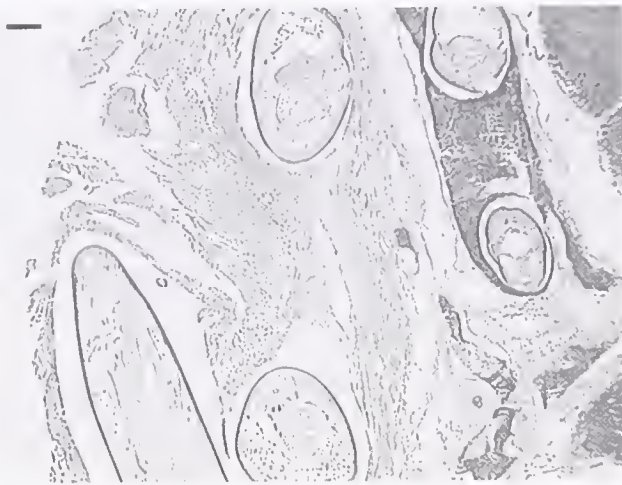


Plate 2. Nematodes parasitizing the male gonad and adjacent tissues of a surf clam. Scale bar is 100 μ m.

1995), but may result from a variety of environmental stressors (e.g., da Ros et al. 1998, Axiak et al. 1988, Marigómez et al. 1990, Gold-Bouchot et al. 1995), although many of these likely also affect nutrition. Winstead (1995), for example, found that poor nutrition was a key element in producing the condition in oysters and that the digestive gland recovered to its normal state relatively rapidly once food supply improved. Therefore, digestive gland atrophy is not necessarily a pathology.

The origin of digestive gland atrophy in surf clams is unknown. Digestive gland atrophy was highest at the more southerly stations (Table 6). Averages at stations 1–3 and 8–9 were above 2 on a 0–4-point scale (Plate 3).

Putative cases of abnormal gonadal development was observed in some surf clams. This set of disorders was characterized by one or more of three conditions. In some cases, gametes developed in an unusual way at the base of the follicles. Follicles sometimes were filled with degenerating gametes and cellular debris beyond the extent normally observed at the end of the spawning cycle (Plate 4, left; Plate 5). Occasionally, immature eggs were observed floating free in the lumen of the follicle (Plate 4, right). The approach used to score instances of abnormal gonadal development was to estimate the fraction of follicles affected, not the degree of effect in each follicle (Table 3). Normally, the entire follicle was completely affected or unaffected. Putative gonadal abnormalities were observed at all stages of the gametogenic cycle (Table 5), so that the possibility that these disorders represent normal phases of gametogenic development seems low. Nevertheless, the origin of the gonadal abnormalities summarized in Table 6 is unknown, as are their statuses as normal or pathologic conditions. Cases of abnormal gonadal development were most common at stations 3, 7, and 8 (Table 6).

Cases of hemocytic infiltration may be focal (localized) (Plate 6) or diffuse (extensive). The type of affected tissue and type of irritation responsible influence the nature of the cellular response (Ford & Tripp 1996). Diffuse infiltration of hemocytes is differentiated from focal infiltration when the affected area does not appear to have a clear center or focal point of highest hemocyte concentration and hemocytes are abundant and distributed broadly over a large section of tissue (Ellis et al. 1998b). In this study, instances of hemocytic infiltration were observed mostly in the connective tissues.

TABLE 6.

Prevalence and infection intensity of the common parasites, pathologies, and tissue disorders observed.*

Station	Nematode Prevalence	Nematode Intensity	Cestode Prevalence	Cestode Intensity	Focal Hemocytic Infiltration Prevalence	Focal Hemocytic Infiltration Intensity	Diffuse Hemocytic Infiltration Intensity	Diffuse Hemocytic Infiltration Intensity	Digestive Gland Atrophy	Abnormal Gonadal Development
1	0.54	4.14	0.85	5.09	0.92	2.75	0.46	1.17	2.13	0.62
2	0.20	2.00	0.47	2.14	0.40	2.00	0.40	1.33	2.40	0.60
3	0.40	2.17	0.67	2.50	0.87	1.46	0.27	1.75	2.25	1.13
4	0.50	3.00	0.50	2.00	0.60	1.17	0.10	1.00	1.50	0.30
5	0.40	3.50	0.50	1.60	0.90	2.00	0.00	0.00	1.60	0.20
6	0.30	2.67	0.80	6.25	0.80	2.25	0.40	1.25	1.90	0.20
7	0.60	2.56	0.87	4.31	0.87	2.46	0.53	1.50	1.80	0.93
8	0.60	2.67	0.80	7.75	0.93	2.29	0.27	1.00	2.14	1.60
9	0.50	2.00	1.00	12.00	0.50	5.00	0.50	1.00	3.00	0.00

*The tissue disorders denoted as digestive gland atrophy and abnormal gonadal development are shown as the mean of their semiquantitative scale values. Calculation of prevalence and infection intensity for the parasites and pathologies is described in the "Materials and Methods" section.

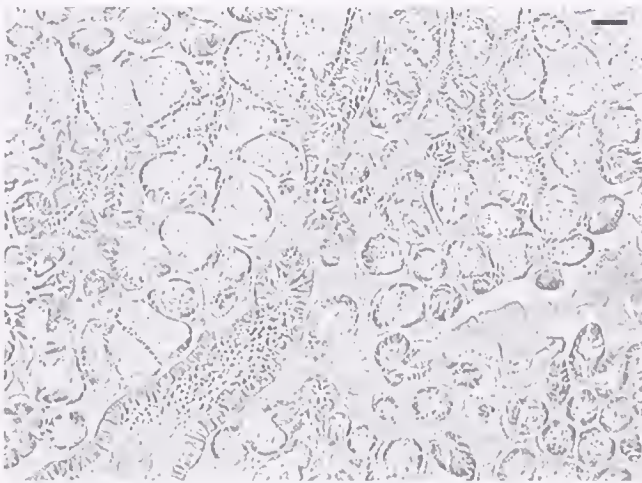


Plate 3. Digestive gland atrophy in a surf clam, scored a 3 on the 0–4-point scale (Table 2). Note the mixture of atrophied tubules extending from the top to the bottom of the plate along the center line and the more normal tubules on the lower right characterized by star-shaped lumens. Scale bar is 100 μ m.

Animals with observed cases of focal hemocytic infiltration were most common at stations 1, 5, and 8 (Table 6); prevalences reached or exceeded 90% at these stations. In contrast, the frequency of focal hemocytic infiltration was highest at stations 1, 7,

and 9, where intensity averaged near or above 2.5 observations per clam tissue subsection. These stations had highest prevalences of nematodes and subsequent statistical analyses support the belief that the two, nematodes and focal infiltration of hemocytes (Plate 6), often are associated. Prevalence of diffuse hemocytic infiltration was highest at stations 1, 7, and 9 (Table 6), where prevalence reached or exceeded 50%. Frequency of occurrence in individual clams, however, was highest at stations 2, 3, and 7. The occurrence of diffuse hemocytic infiltration was not obviously associated with either of the common parasites, nematodes or cestodes.

Association of Parasites, Pathologies, and Other Indicators of Health

We assumed that condition index was a good overall indicator of animal health and examined the relationship between condition index and the common parasites, pathologies and other tissue disorders. We focused on infection intensity, rather than prevalence. Condition index is the ratio of wet weight to length. However, this ratio is not constant over all size classes. Typically, the shell growth form changes with age, such that shell width increases disproportionately with shell length. As a consequence, larger clams typically have a disproportionately larger weight per centimeter of length, and, accordingly, condition index tends to average higher. Preliminary analyses confirmed that some histopathological variables were better explained by shell length than by weight or condition, as a consequence of the dichotomous nature of length. For this reason, we first used principal components analysis

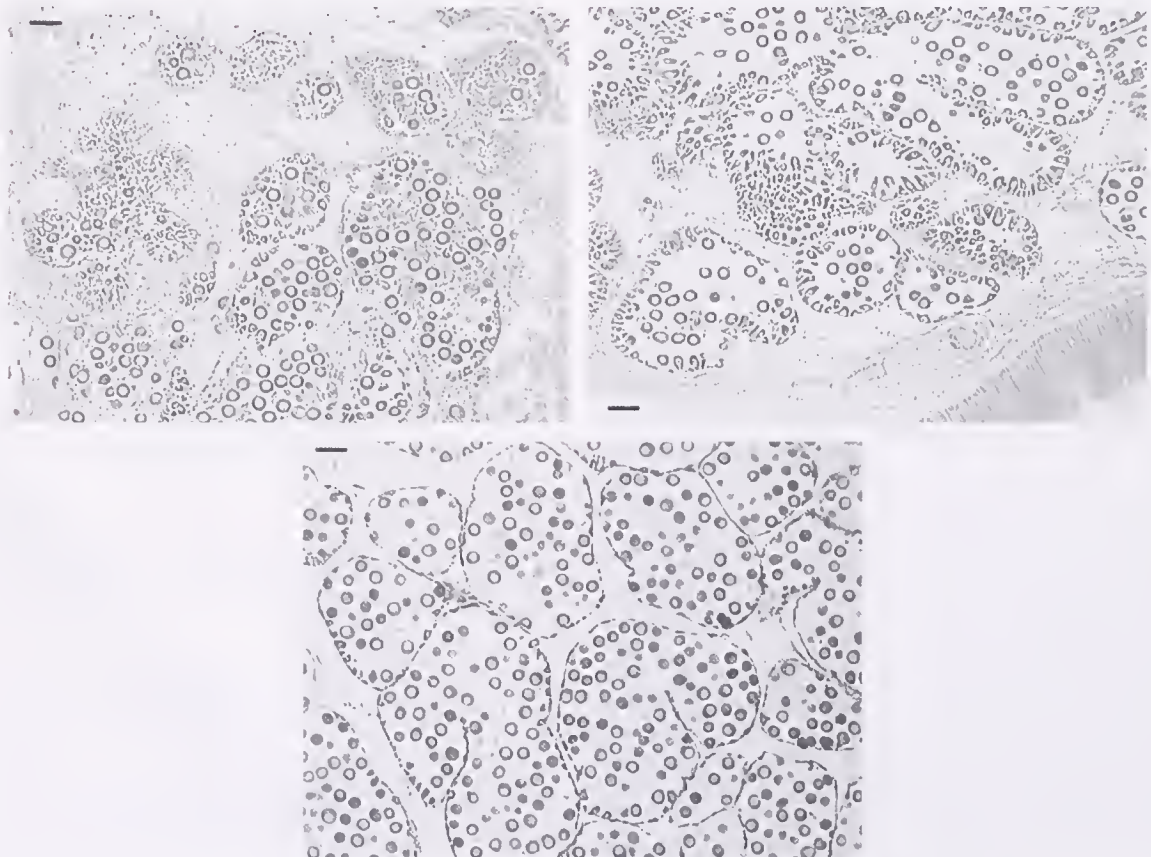


Plate 4. Putative gonadal abnormalities in female surf clams. Upper left: A section through an S3-stage gonad (Table 4) showing degenerating eggs and cellular debris among unspawned, fully mature eggs. Upper right: A section through an S3-stage gonad (Table 4) showing immature eggs floating free in a follicle with mature eggs (note the follicles in the center of the field). Lower: A section through a normal S3-stage gonad. Scale bar is 100 μ m.

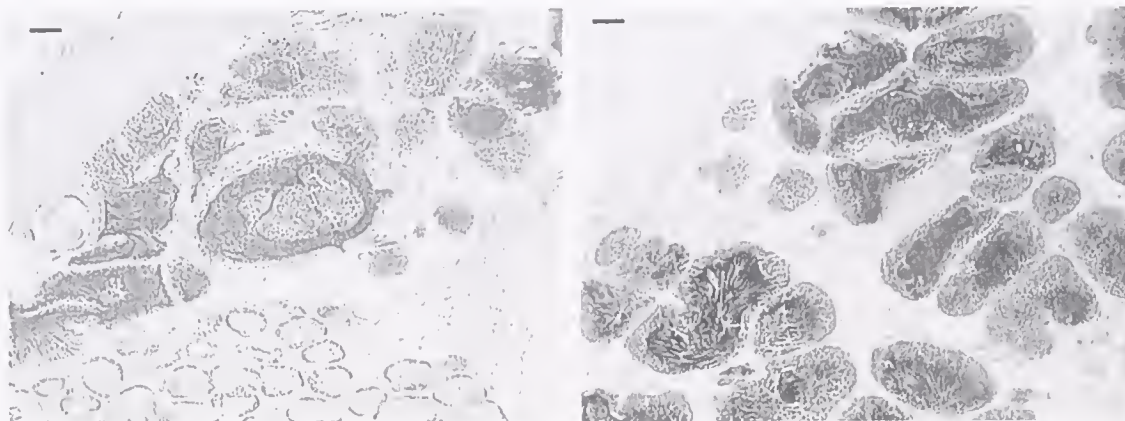


Plate 5. Left: Putative gonadal abnormality in a male surf clam, showing a degenerating follicle within a gonad otherwise rated stage D3 (Table 4). Right: A section through a normal D3-stage gonad. Scale bar is 100 μ m.

(PCA) to separate the simple relationship between weight and length, encompassed in the measure of condition, from the additional effect of length, presumably reflecting the change in allometric growth form. This PCA provided two significant factors. Factor 1, hereafter referred to as the condition factor, combined the simple measurement of weight with condition index. The variable length was split, as anticipated, relatively evenly between factors 1 and 2, the latter hereafter referred to as the additional length factor.

We also used PCA analysis to generate variables describing the relationships of the various parasites, pathologies, and other tissue disorders because preliminary analyses indicated that many pairwise correlations were significant (Spearman's rank correlation, $\alpha = 0.05$). Three PCA factors were significant. Factor 1, hereafter referred to as the nematode factor, combined the variables of nematode infection intensity and the frequency of focal hemocytic infiltration, thereby supporting a relationship between this parasite-tissue pathology pair. Factor 2, hereafter referred to as the abnormality factor, was principally determined by the mean intensity of gonadal abnormality scores. Cestode infection intensity loaded about evenly between these first two factors, probably because cestode prevalence and cestode infection intensity tended to be somewhat differentially distributed (Table 6). Factor 3, hereaf-

ter referred to as the atrophy factor, was primarily determined by the degree of digestive gland atrophy. Diffuse hemocytic infiltration did not contribute significantly to any of these three PCA factors.

ANOVAs were run using the condition and length factor scores as dependent variables, the nematode, abnormality, and atrophy factor scores as independent variables, and using sex and gametogenic stage compressed into the three categories of developing gonad, ripe gonad, and spawning or partially spent gonad as added main effects. The condition factor was significantly influenced by the abnormality factor ($P = 0.0038$). Low condition indices were associated with high levels of abnormal gonadal development (Fig. 2). Cestode infection intensity also loaded on this factor, and the highest cestode infection intensities were associated with a small reduction in condition index (Fig. 2), however, not to the extent observed for gonadal abnormalities (Fig. 2).

The length factor described that part of the variation in length not encompassed by the simple relationship of weight and length as expressed by condition. The length factor was significantly influenced by the degree of digestive gland atrophy, as expressed by the atrophy factor scores ($P = 0.015$). Highest digestive gland atrophy scores occurred in the smallest animals (Fig. 3). The same trend was present with condition index (Fig. 3), but this trend was not statistically significant when analyzed by comparison of the appropriate PCA scores. The main effects of sex and gametogenic stage were not significant.

The nematode factor, representing nematode infection intensity and the frequency of focal hemocytic infiltration, and partially explaining cestode infection intensity, did not significantly influence either the condition or the length factor (Fig. 4), nor were the main effects of sex or gametogenic stage significant.

We reserved the analysis, taking the nematode, abnormality, and atrophy factors as dependent variables and the length and condition factors as independent variables to determine the degree to which condition, length, gametogenic stage, or sex influenced the occurrence of parasites and pathologies. Sex and compressed gametogenic stage were again included as main effects. Not surprisingly, the atrophy factor as a dependent variable and the length factor as an independent variable were significantly related ($P = 0.0074$). The intensity of digestive gland atrophy varied with clam length (Fig. 3). The atrophy factor was not significantly influenced by sex nor, as might be expected, was it significantly influenced by gametogenic stage, or the condition factor. The abnormality factor,

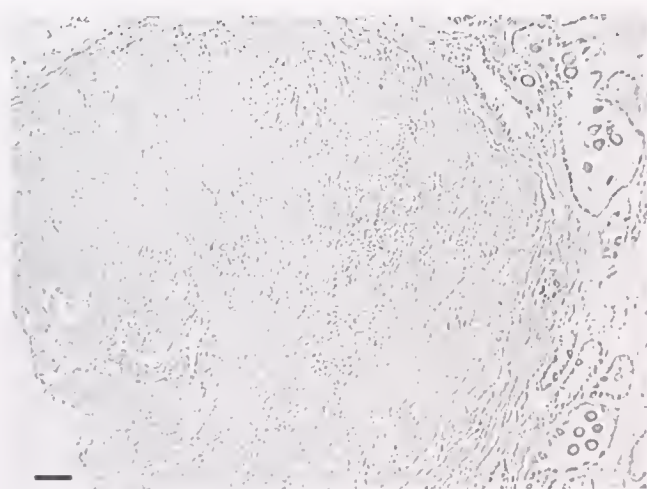


Plate 6. Focal infiltration of hemocytes in the region of the female gonad of a surf clam, likely resulting from nematode infection. Scale bar is 100 μ m.

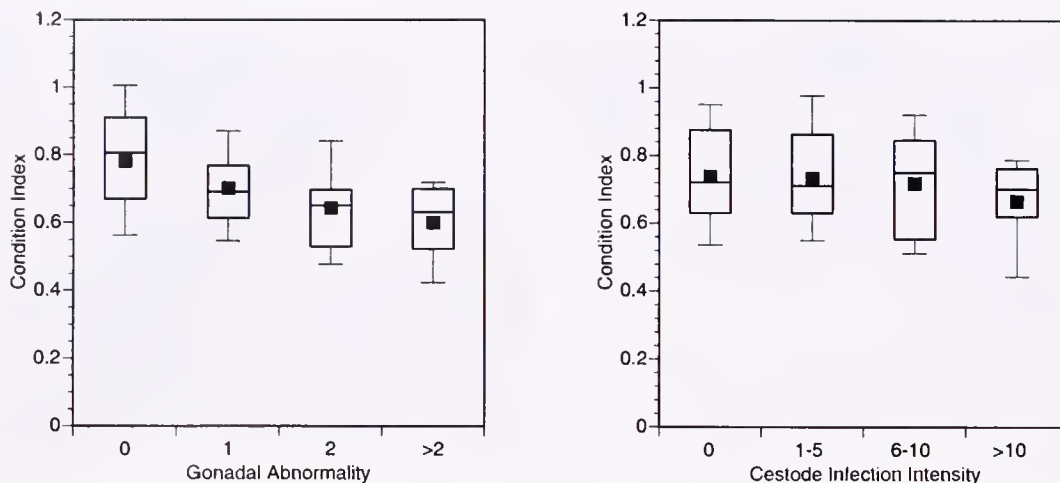


Figure 2. Left: Box plot relating condition index (g mm^{-1}) to the severity of abnormal gonadal development (scores defined in Table 3) in surf clams. Right: Box plot relating condition index (g mm^{-1}) to cestode infection intensity expressed as the number of observations per clam subsection in surf clams. Boxes encompasses the 25th and 75th percentiles with the median as the dividing line. The whiskers identify the 10th and 90th percentiles. The mean is indicated by a ■.

that expressed the severity of gonadal abnormality and, to a certain extent, cestode infection intensity, was significantly influenced by the condition factor ($P = 0.0034$). Higher gonadal abnormality scores occurred in animals with lower condition index (Fig. 2). Gametogenic stage also significantly influenced the abnormality factor scores ($P = 0.02$). An *a posteriori* LS means test revealed that gonadal abnormalities, as expressed by the factor scores, were more severe within gametogenic stages in which evidence of spawning was present (S stages, Table 4) than with stages of gamete development (D stages, Table 4) ($P = 0.03$) or ripe gonads without evidence of spawning (stage 5, Table 4) ($P = 0.0074$). Animals rated as ready to spawn had lower gonadal abnormality scores than those in which some evidence of the initiation of spawning was present (Fig. 5). Cestode infection intensity varied little with gametogenic stage (Fig. 5), indicating that the primary effect determining the relationship between the abnormality factor and gametogenic stage was the degree of gonadal abnormality.

Factor one, jointly describing nematode infection intensity and

the frequency of focal hemocytic infiltration, plus some portion of cestode infection intensity, was significantly influenced only by sex. Nematode factor scores were higher in males than in females ($P = 0.043$), as were nematode infection intensities (Fig. 6). The frequency of focal infiltration of hemocytes was also elevated, although to a lesser degree in males (Fig. 6).

DISCUSSION

We investigated the health of surf clams taken along the "mortality" line running southeast off the Delmarva Peninsula that separates stations that yielded no living surf clams in the 2002 NMFS survey (NEFSC 2003) from those where living surf clams were collected. Condition index may serve as a good overall indicator of animal health, once gametogenesis is taken into account, as condition normally varies with the gametogenic cycle (Beninger & Lucas 1984, Choi et al. 1993, Choi et al. 1994, Loesch & Evans 1994). Condition declines for many reasons, including low nutri-

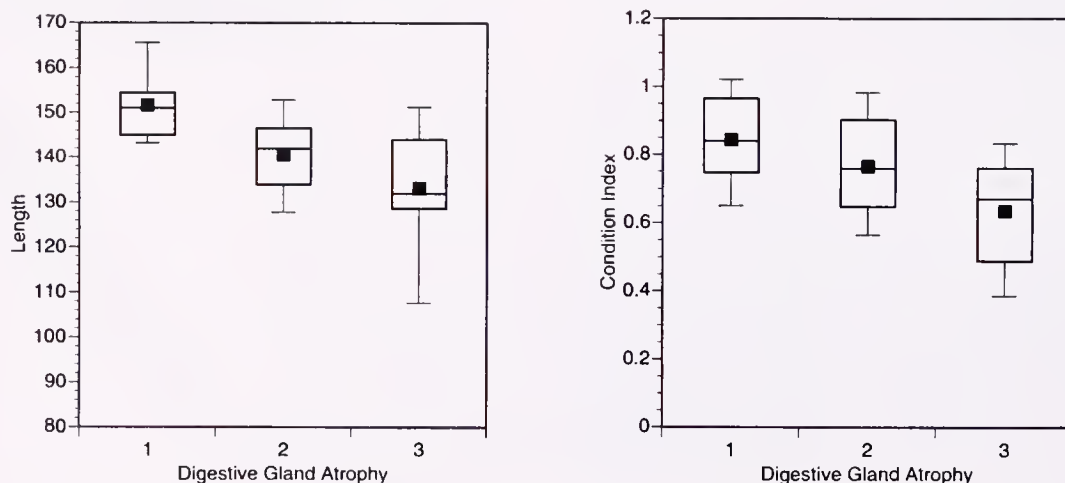


Figure 3. Left: Box plot relating length (mm) to digestive gland atrophy (scores defined in Table 2) in surf clams. Right: Box plot relating condition index (g mm^{-1}) to digestive gland atrophy (scores defined in Table 2) in surf clams. Boxes encompasses the 25th and 75th percentiles with the median as the dividing line. The whiskers identify the 10th and 90th percentiles. The mean is indicated by a ■.

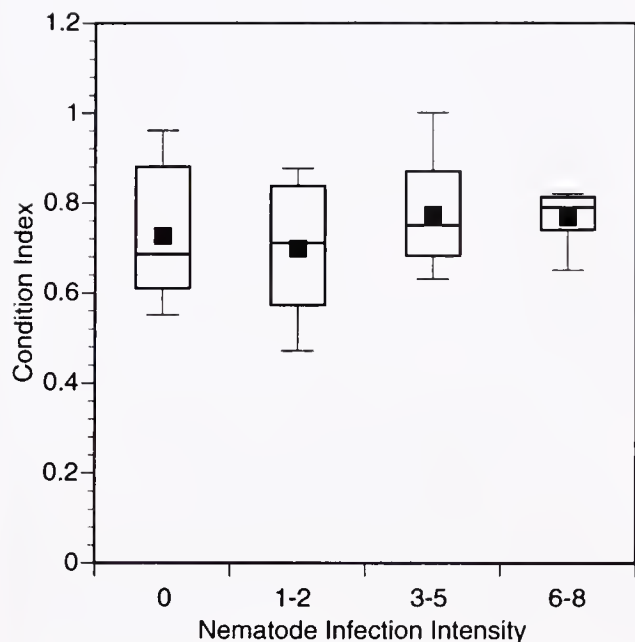


Figure 4. Box plot relating condition index (g mm^{-3}) to nematode infection intensity expressed as the number of observations per clam subsection in surf clams. The box encompasses the 25th and 75th percentiles with the median as the dividing line. The whiskers identify the 10th and 90th percentiles. The mean is indicated by a ■.

tion produced by restricted food supply (Engle & Chapman 1953, Deslous-Paoli & Héral 1988, Bielefeld 1991, Rheault & Rice 1996, Honkoop & Beukema 1997, Smith et al. 2000, Krauter et al. 2003) and by the influence of parasites and disease (Plana et al. 1996, Pérez Camacho et al. 1997, Olivas-Valdez & Cáceres-Martínez 2002, Hine 2002).

The most northern stations, 4, 5, and 6, had the highest condition indices, the lowest gonadal abnormality scores, with one exception, and the lowest digestive gland atrophy scores. Animals at the stations with lowest average condition index had body weights less than 65% of the mean animal taken at station 6 with the highest condition index. We assume, from Weinberg et al. (2002), that surf clams at station 6 already averaged lower in condition index than clams from more northern climes, as condition index is normally lowest off the Delmarva Peninsula. The condition indices we recorded from clams at the southernmost stations occupied in this study were extremely low in comparison to values recorded by Loesch and Evans (1994) for the nadir of the normal seasonal cycle. Loesch and Evans (1994) recorded a drop in condition index of 20–25% in the late fall from summertime highs. Though exceptions exist (e.g., Héral & Deslous-Paoli 1983, Garton & Haag, 1993), most bivalves lose about 20–25% of their body weight upon spawning (Browne & Russell-Hunter 1978, Powell & Stanton 1985, Choi et al. 1994, DiBacco et al. 1995), so the observations of Loesch and Evans (1994) are typical of bivalves.

Larger reductions in condition index in bivalves, of the order observed at the more southern stations in this study, are often related to nutritional challenge. Pérez Camacho et al. (1997), for example, record an approximately 50% decline in condition in parasitized *Mytilus edulis*. Beninger and Lucas (1984) record reductions in excess of 50% in *Tapes* clams during periods of reduced food supply, particularly over winter. Similar results are reported by Honkoop and Beukema (1997), Olivas-Valdez and

Cáceres-Martínez (2002), Barber et al. (1988), and others. Although a number of experiments subjected bivalves to long-term deprivation of food (e.g., Holland & Spencer 1973, Riley 1976, Riley 1980, Hawkins et al. 1985, Bielefeld 1991, Chase & McMahon 1994, Hummel et al. 1995), all of which show decreases in condition or body components related to condition, the degree to which bivalves can recover from a 30% to 50% loss of somatic tissue is poorly known. Leighton and Boolootian (1963) recorded one of the few measures of mortality due to starvation. They report that abalone began to die after a weight loss of 12–24% over a two- to three-month time period. Nevertheless, condition indices much below 30% of normal, as observed in this study at the southernmost stations, particularly during the summer months when condition should be at or above the long-term mean value (Loesch & Evans 1994), strongly imply that malnourished clams were common along the “mortality” line.

Additional evidence of malnutrition accrues from the tendency for stations yielding clams in lowest condition to also yield clams with above normal scores for abnormal gonadal development. In studies of the surf clam gametogenic cycle, neither Ropes (1968), Jones (1981), nor Chintala and Grassle (1995) figure gonadal conditions of this type. Although the origin of the gonadal abnormalities observed in these clams is unknown, as is the accuracy of their designation as abnormal, an unusual degree of gonadal resorption or loss of gonadal integrity does seem a likely cause. Gonadal resorption often occurs at the end of the gametogenic cycle (e.g., Ropes 1968, Griffiths 1977, Chung & Kim 1994, Ellis et al. 1998a), but resorption is also a frequent indicator of malnutrition (Riley 1976, Bielefeld 1991, Hofmann et al. 1992, Barber 1996, Delgado & Pérez Camacho 2003). The correlation between low condition index and higher degrees of gonadal abnormality in this study suggests that the latter originates in the same causative factors that produce the unusually low condition indices.

Digestive gland atrophy also is normally associated with factors compromising nutritional status. In this study, highest scores of digestive gland atrophy tended to occur in smaller animals (Spearman’s rank correlation, $P = 0.0045$). Smaller animals were collected at the more southern stations and these stations also were characterized by low condition index. Why digestive gland atrophy should be more significantly influenced by the additional length factor rather than the factor encompassing weight and condition is unknown.

A syndrome would appear to emerge from this data analysis. Animals with low condition are often animals with higher scores of gonadal abnormality and higher scores of digestive gland atrophy. Among the factors often associated with reduced condition, as well as impacting gonadal development (e.g., Powell et al. 1999, Arnold et al. 2002, Park et al. 2003), is parasitism and disease. Two parasites were commonly observed in the surf clams: nematodes and cestodes. Parasites of this type are rarely associated with overt pathologies, beyond the frequently observed inflammatory response characterized by focal hemocytic infiltration. Infection intensities were routinely higher than recorded in mussels and oysters from the east coast, however (Kim et al. 1998). Two pathologies were also recognized, focal and diffuse hemocytic infiltration. One, focal infiltration of hemocytes, would appear to be related to the presence of worms, particularly parasitic nematodes.

Neither of the two parasites nor either of the pathologies, however, was significantly correlated with condition index or length, as expressed by comparisons between the appropriate PCA factor scores, save for the influence of cestode infection intensity in the

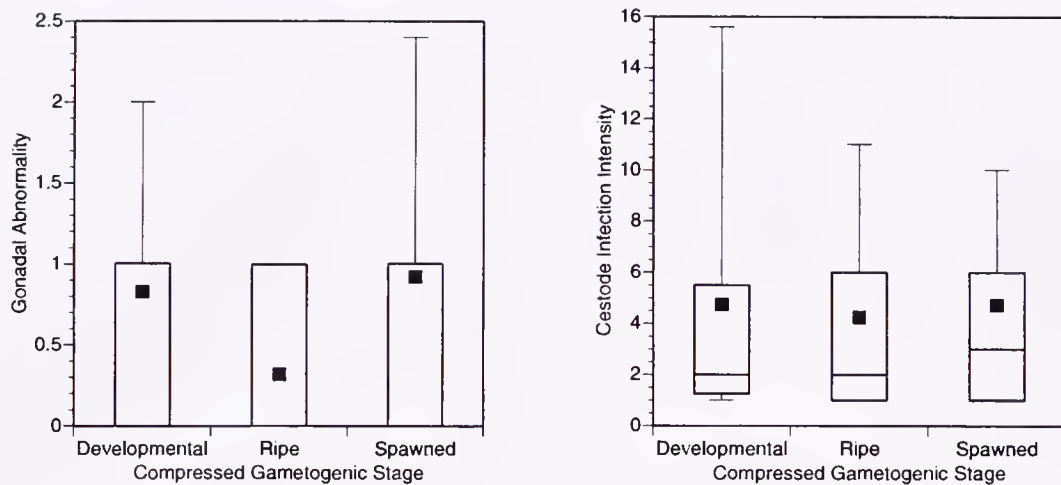


Figure 5. Left: Box plot relating the severity of gonadal abnormality (scores defined in Table 3) to gametogenic stage (defined in Table 4) in surf clams. Right: Box plot relating cestode infection intensity expressed as the number of observations per clam subsection to gametogenic stage (defined in Table 4) in surf clams. The box encompasses the 25th and 75th percentiles with the median as the dividing line. The whiskers identify the 10th and 90th percentiles. The mean is indicated by a ■. For the gametogenic stages designated "Spawned" and "Developmental" on the left graph, and median and 75th percentile are equivalent; for the gametogenic stage designated "Ripe," the median and 25th percentile are equivalent, as are the 75th and 90th percentiles.

PCA factor principally describing the occurrence of gonadal abnormality. By comparison, the PCA factors representing the disorders denoted as gonadal abnormality and digestive gland atrophy significantly influenced one or both of the condition and length factors. Nematodes and the frequently associated cases of focal hemocytic infiltration were not obviously more common in the southern stations where condition index was low. Cestodes tended to be more common at sites where condition index was low, but not consistently so. Infection intensity was high at station 6, for example, where the highest condition index was also recorded. One cannot fully exclude disease as an underlying cause of the malnourished state, however, although no disease-causing organisms were observed in this study. Many disease-causing organisms are readily identified, but cases that are otherwise, such as the withering syndrome of abalones in which the etiological agent was not easily confirmed (VanBlaricom et al. 1993, Friedman et al.

1993, Moore et al. 2001, Moore et al. 2002), are well-known. Nevertheless, parasites and disease do not seem to be likely mediators of the syndrome that appears to evince a malnourished state observed in surf clams along the mortality line.

Sex and gametogenic stage also offered little explanatory information. Sex was unrelated to any condition save possibly the presence of nematodes. Gonadal abnormalities were more common in clams with gonads that showed evidence of the initiation of spawning, suggesting that abnormal development may become more likely or more recognizable as the gametogenic cycle progresses. Digestive gland atrophy and condition index were not so associated. Many bivalves, even when malnourished, attempt to complete gametogenesis (e.g., Riley 1976, Bielefeld 1991, Delgado & Pérez Camacho 2003), though not necessarily successfully, and surf clams seem to be no exception, based on the limited information presented here.

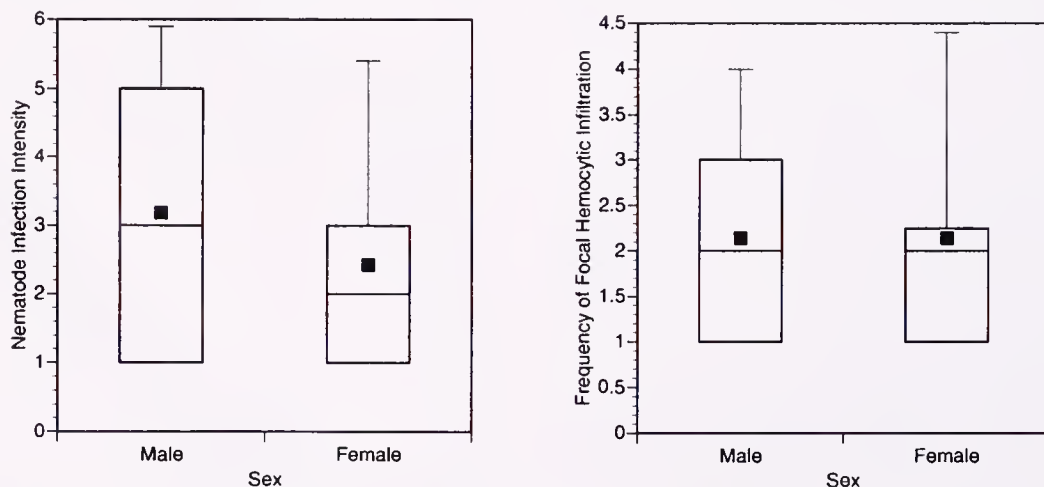


Figure 6. Box plot relating nematode infection intensity expressed as the number of observations per clam subsection on the left and the frequency of focal hemocytic infiltration per clam subsection on the right to sex in surf clams. The box encompasses the 25th and 75th percentiles with the median as the dividing line. The whiskers identify the 10th and 90th percentiles. The mean is indicated by a ■.

Ready alternatives as causative factors producing a malnourished state do exist, however. A rise in temperature can restrict scope for growth, particularly in the temperature range above optimal where respiratory rate continues to increase, but filtration rate begins to decline (Ali 1970, Newell et al. 1977, Winter 1978, Newell & Branch 1980, Brock & Kofoed 1987). Variations in climate and oceanographic conditions might also directly influence food supply (e.g., Lehman 2000). The combination of rising temperature and reduced food for such large animals as surf clams requiring substantial food resources to maintain their bulk could be lethal (Taylor 1960, Powell et al. 1995). Unfortunately, sufficient information is not available to investigate further the origin of the malnutrition observed. The evidence does suggest, however, that the regional mortality event observed by NEFSC (2003) in 2002 may be continuing, that animals in nutritionally limiting situations continue to exist over a wide area off the Delmarva Peninsula, and that many of these animals are in sufficiently poor condition that recovery is not necessarily assured, should the environmental conditions leading to malnourishment relax.

The evidence suggests, therefore, that factors compromising scope for growth, either a direct reduction in food, factors reducing the ability to acquire food, or factors increasing the energy requirements of maintenance, offer potential as the underlying cause of malnutrition. Range shifts are often determined by extreme events,

particularly along the trailing edge (e.g., Taylor 1934, Kennedy 1990). Range expansion occurs by recruitment. Range contractions in short-lived animals can occur by the failure thereof. For long-lived animals, such as surf clams, rapid contractions in range require increased adult mortality along the trailing boundary and such contractions may occur more rapidly than the expansion along the leading edge (Kennedy 1990). Starvation brought on by environmental shifts mismatching food supply and feeding rate with the energy demands of tissue maintenance offers one potential way this might occur. The malnourishment syndrome identified here, low condition index associated with increased frequency of gonadal abnormality and increased levels of digestive gland atrophy, may evidence such a process.

ACKNOWLEDGMENTS

The authors thank the captain and crew of the *F/V Betty C.* for help in sample collection and J.H. Miles & Co., Inc., for providing the vessel. We appreciate the help of J. Weinberg in providing station locations and catch statistics from the 2002 NMFS-NEFSC survey. This research was supported by the National Fisheries Institute Clam Committee and the North American Clam Association. We appreciate the support and logistical help from both chairmen, Dave Wallace (NACA) and Daniel Cohen (NFI-CC).

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REPRODUCTIVE CYCLE OF THE STOUT RAZOR CLAM, *TAGELUS PLEBEIUS* (LIGHTFOOT, 1786), IN THE MAR CHIQUITA COASTAL LAGOON, ARGENTINA

M. CLEDÓN,^{1,2,*} A. C. PERALTA BRICHTOVA,³ J. L. GUTIÉRREZ⁴ AND P. E. PENCHASZADEH¹

¹FCEyN-Universidad de Buenos Aires, MACN-CONICET, Argentina; ²Alfred Wegener Institute, Bremerhaven, Germany; ³Facultad de Ciencias Naturales y Museo de la Plata, Argentina;

⁴Universidad de Mar del Plata, Argentina

ABSTRACT A population of the dioecious euryhaline bivalve, *Tagelus plebeius*, living in the Mar Chiquita Lagoon (37°40'S, 57°26'W) exhibits an annual gonad cycle. Spawning occurs during the austral spring and summer (October to March) and is dependent on water temperature. Two reproductive modes were observed during consecutive years: partial spawning events in December 1999 to January 2000 and at the end of March 2000, and one continuous spawning event in October 2000 to March 2001. Mature oocytes measured 75–95 μ m. At the end of the spawning events there was a sharp drop in body weight, indicating that body mass is determined by gonad status.

KEY WORDS: razor clam, reproduction, *Tagelus*

INTRODUCTION

The Psammobiid euryhaline bivalve *Tagelus plebeius* (Lightfoot 1786) inhabits tidal flats on the Atlantic coast of the Americas (Osorio Ruiz 2002) from 34°N to 41°S, contributing more to the total biomass of these areas than any other bivalve. It is such a common inhabitant of the estuaries and coastal lagoons of Argentina, that shell beds of this species modify the environment (Gutiérrez & Iribarne 1999). Individuals live buried in sandy substrates, and can tolerate a wide range of salinities (<10‰ to >30‰) and temperatures (Chanley & Castagna 1971).

Most *Tagelus* species, including *T. plebeius*, are economically important as a source of food in Chile, China, Spain, and other countries, with the harvest of more than 4000 tons per year in Chile alone (Osorio Ruiz 2002). Some information is available on the biology of other *Tagelus* species such as, *T. dombeii* (Urban 1996) and *T. divisus* (Fraser 1967). Blommer (1907) described the anatomy of *T. gibbus* and *T. divisus*, and *T. dombeii* has been used as an indicator of contamination in marine environments (De Gregori et al. 1994). Despite its economic importance, there are some large gaps in our knowledge of the biology of *T. plebeius*. Here we present a study of the reproductive cycle of a population in Mar Chiquita Lagoon (Buenos Aires, Argentina).

MATERIAL AND METHODS

From September 1999 to August 2001, 20 adult *T. plebeius* (more than 40 mm in shell length) were collected every month with a shovel. The sampled population is located in the middle zone of the Mar Chiquita Lagoon. The maximum length of each specimen was measured to the nearest mm with a digital vernier calliper. The edible part was removed from the shell, weighed to the nearest mg, fixed in Bouin's solution for 72 h, preserved in 70% alcohol, dehydrated in ethanol series and infiltrated with paraffin. For histologic analysis, gonad sections of 5 μ m thickness were stained with Mayer's hematoxyline and eosin. About 10 females of each sampled month were analyzed under a Zeiss microscope. Of each female the diameter of 10 unbroken oocytes, showing nucleoli were measured from 5 sections. These sections were

anatomically distant from each other to avoid repetition of measurements.

Surface water temperature was measured with a mercury bulb thermometer four times a day on 3 consecutive days of each month to calculate the daily mean temperature of the month. The mean water temperature and its standard deviation (SD) were plotted together with the gonadic stage and wet weight of the individuals.

RESULTS

No Hermaphroditism or Sex Reversal Was Encountered

Sex can be distinguished only histologically and presents a ratio of male to female not significantly different from 1:1 ($\chi^2 = 0.52$, $P > 0.05$).

The diameter of the oocytes (Fig. 1) and mean body mass (Fig. 2) were correlated with water temperature (Fig. 3), with significant decreases at the beginning of spawning and reabsorption from October 1999 to January 2000 (ANOVA, $P < 0.05$), May to July 2000 (ANOVA, $P < 0.05$) and November 2000 to March 2001 (ANOVA, $P < 0.05$).

No Asynchronous Gametogenesis Was Observed

Figure 1 shows how mean oocyte diameter increases, whereas the SD remains more or less constant, during winter maturation. Before spawning begins, all oocytes are mature, mean oocyte diameter is at its highest with a small SD. Once spawning begins, the mean oocyte diameter decreases and the SD increases, because a new cohort of oocytes simultaneously starts to grow. As water temperature rises in the early spring, body mass increases and gametes start developing (Fig. 4), reaching their maturity (Fig. 5) in October. During 1999, the main reproductive event occurred between December and January, when modal oocyte diameters were 75–95 μ m and water temperature in the lagoon was about 20°C. This event was characterized by a nearly complete evacuation of the gonads (Fig. 6) and a sharp drop in shell free wet mass (SFWM) to the annual minimum. After this event, new gametes developed and body mass increased again rapidly. Another spawning event occurred at the end of March 2000 with a similar body mass fluctuation.

During the period of sexual activity, non-released gametes co-exist with developing ones. When the water temperature dropped

*Corresponding author. E-mail: mcledon@bg.fcen.uba.ar

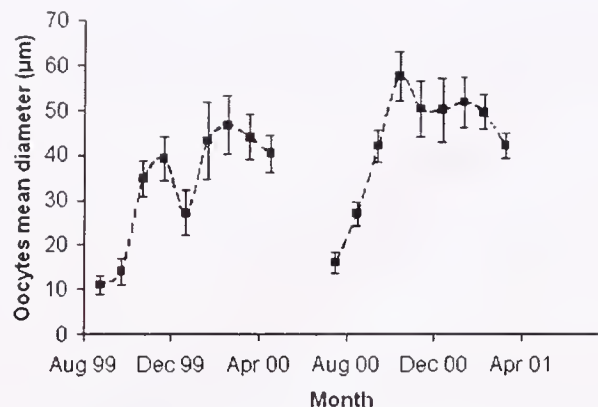


Figure 1. Mean oocyte diameter, and standard deviation, of *Tagelus plebeius* from Mar Chiquita Lagoon. The data gap in June to July 2000 and May to August 2001 is due to the ovary reabsorption phase showing now measurable oocytes. $n = 1826$.

below 20°C, gonad reabsorption started (Fig. 7) and SFWM decreased. By winter, gonads were completely reabsorbed.

During the year 2000, the population showed a different reproductive strategy, characterized by a progressive spawning event that lasted the entire summer, from October 2000 to March 2001. The modal diameter of oocytes during December 1999 was approximately 50 µm because of a lack of small oocytes, and 70 µm in December 2000.

DISCUSSION

We observed two different spawning modes in *T. plebeius*. The gonads can either be emptied completely in a single event, or oocytes can be retained for later release during various partial-spawning events throughout the summer.

After a complete spawn, gonad recovery was immediate, allowing a second spawning event during March. A similar pattern has been observed in a population of *Donax trunculus*, along the coast of Portugal, with one major spawning event at the beginning of spring and the other lasting the whole summer (Gaspar et al. 1999). Tirado & Salas (1998) reported two peaks of gametes release in *D. trunculus*, in May and August (septentrional summer). Our study also agrees with the report of Chanley and Castagna (1971) on *T. plebeius*, where they reported individuals spawning in laboratory at the end of the winter and again mature gametes in

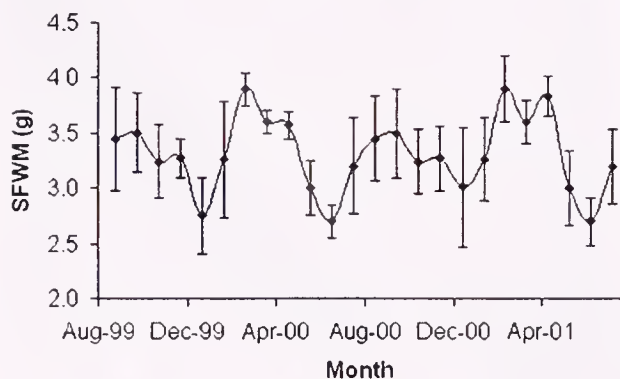


Figure 2. Seasonal variation in shell free wet mass (SFWM) of 50–60 mm of *Tagelus plebeius* from Mar Chiquita Lagoon. $n = 480$.

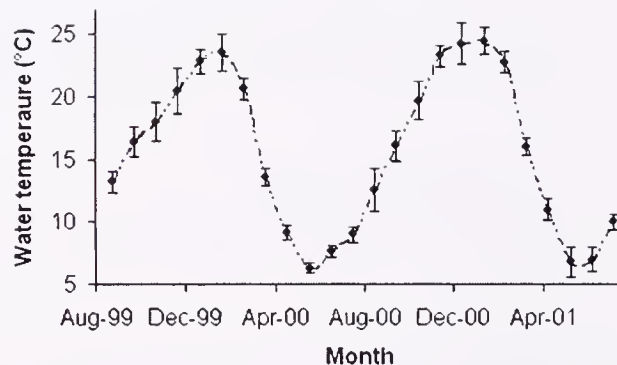


Figure 3. Mean water temperature and Standard Deviation in Mar Chiquita Lagoon.

summer. Another case of partial spawning events was observed in a population of *Tellina petitiiana* in Golfo Nuevo, Argentina (Baron & Ciocco, 2001). First partial spawning was registered in November (late spring) and from December to February most of the clams were in the partial evacuation and recuperation stages. This spawning strategy coincides with the data presented here for *T. plebeius*.

Dekker & Beukema (1999) reported a fluctuation in the reproductive success of populations of *Tellina tenuis* and *Abra tenuis* in the north of their geographical range, depending on summer and winter water temperatures. This can also occur in *T. plebeius*, where temperature can influence the start of gametes development and spawning. Variation in environmental conditions cannot only produce inter-annual changes in a population's reproductive strategy, as we report here, but also in the same year among populations. Tirado & Salas (1998) reported reproduction of *D. trunculus* from February to October in a Spanish Mediterranean population, whereas Gaspar et al. (1999) reported reproduction from March to August in a Portuguese population at the same latitude. If the population does not change its strategy, its recruitment success

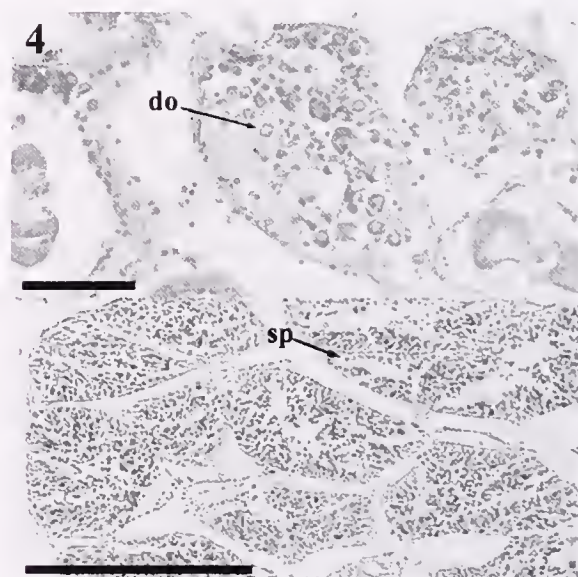


Figure 4. *Tagelus plebeius* developing female and male gonads. Developing oocytes (do) in female gonad. Scale bar: 100 µm. *Tagelus plebeius*. Developing spermatides (sp) in male follicles. Scale bar: 500 µm.

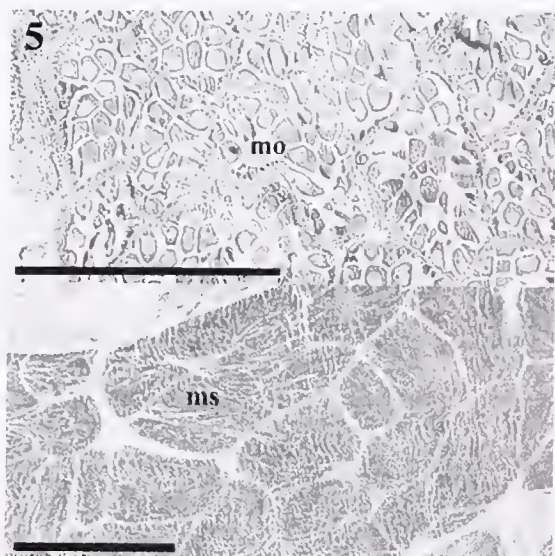


Figure 5. Completely developed female and male gonads in *Tagelus plebeius*. Mature oocytes (mo). Scale bar: 400 μ m.

could be affected, as reported for *T. tenuis* in Dublin bay by Wilson (1997). This shifting effect is more evident when comparing populations from different latitudes where a more continued and less marked reproductive season near the Equator contrasts with biannual cycles in higher latitude populations. This phenomenon is registered for many species and probably it occurs in other *T. plebeius* populations.

So, in these cases temperature dependency can explain the changes in reproductive strategy as suggested by Dekker & Beukema (1999).

Piccolo & Perillo (1997) reported water temperature in Mar Chiquita between 9 °C and 25°C for the period 1994 to 1995. This is a moderated fluctuation compared with the 4.5°C to 27°C registered in the present work. These differences surely influence the reproduction of this population. In this sense it would be necessary to study how the population dynamics change under different environmental conditions.

Salinity was not registered in our study; however, there is a longitudinal gradient between the mouth of the lagoon (34‰), the middle zone (22‰), and the streams entrances (3‰ to 4‰) with a very homogeneous vertical distribution (Piccolo & Perillo 1997). Because we have no available data on salinity, we inferred a more or less constant salinity of 22‰ in the sampled area.

T. plebeius is the only present bivalve in this community. The recruitment of this population has not been studied here, but it

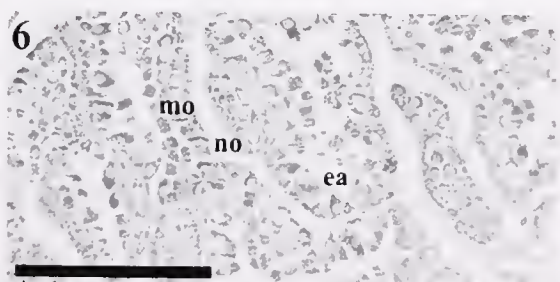


Figure 6. Partially spawned female gonad in *Tagelus plebeius* with some empty areas (ea), mature oocytes (mo) and new developing oocytes (no). Scale bar: 500 μ m.

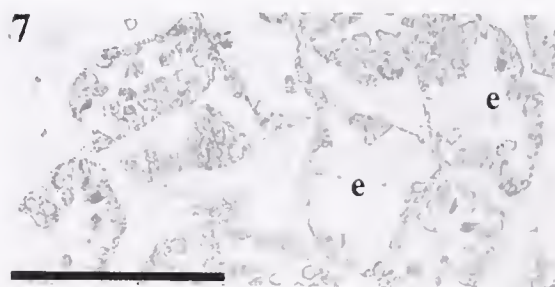


Figure 7. *Tagelus plebeius* female gonad after spawning presenting conspicuous empty spaces (e) and a less compressed gonadic anatomy. Scale bar: 600 μ m.

could be only influenced by the population density, as reported by Stephen (1938) for *T. tenuis*, where it attains high population densities in spite of sporadic recruitment, coincides with high environmental temperatures, and probably an overall improvement in conditions.

The seasonal weight changes of *T. plebeius* individuals correspond with their reproductive cycle. This observation can be also related to the fact that euryhaline bivalve species commonly show a pattern of two spawning peaks per reproductive season, as reported for *Abra alba* (Hily & Le Bris 1984). A body mass cycle governed by the gonad cycle has been observed in other commercial species of the genus, as *T. dombeii* (Urban 1996) and *T. divinus* (Fraser 1967). Urrutia et al. (2001) reported high lipid content in a Chilean *T. dombeii* population during October and February, at the time when gonad weight is higher, this could explain the increment in body mass when spawning approaches.

Tagelus plebeius is a good candidate for managed exploitation or aquaculture for the following reasons: because other *Tagelus* species are commercialized (Urban 1996 Osorio Ruiz 2002) its similarity to the other commercial species ensures its insertion in an existing market.

The stock can be managed quite easily because they inhabit coastal lagoons that can be more easily monitored and protected in comparison with open waters species. Moreover, the coast of the province of Buenos Aires is formed by a stable paleodunes field (Codignotto & Aguirre 1993) where artificial coastal ponds could be constructed for its farming at low cost.

Animals of Marketable Quality May Be Obtained Over Several Months

Studies on the populations growth rate, size at sexual maturity, and evaluation of the stock have to be carried out, as well as the influence of environmental changes on the populations dynamics, to complete the knowledge on this species before starting its commercial exploitation.

ACKNOWLEDGMENTS

We are grateful to Dr. G. Darrigran and Dr. C. Damborenea for their help in processing the material. Dr. M. von Hedor and J. P. Jiede kindly helped with the laboratory procedures. We would like to thank Susan M. Gaines for revising this paper. M. Cledón wants to thank to Comisión de Investigaciones Científicas of Buenos Aires and DAAD for partial financial support during the development of this work. We also thank Guillermina Cosulich and Gabriela Silvoni for providing literature. This research was partially funded by UBACYT \times 316, PIP 02222222193 and P-2002-01-10975.

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REGENERATION TIME AND MORPHOLOGY OF THE INHALANT SIPHON OF *DONAX DENTICULATUS* LINNAEUS, 1758 (BIVALVIA, DONACIDAE) AFTER AMPUTATION

PATRICIA MILOSLAVICH,¹* PABLO E. PENCHASZADEH,²* ANA KARINNA CARBONINI¹
AND DIEGO SCHAPIRA¹

¹Universidad Simón Bolívar, Departamento de Estudios Ambientales, P.O. Box 89000, Caracas, 1080, Venezuela and ²Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Biología, Laboratorio de Invertebrados, Ciudad Universitaria, Pab. II, C1428EHA, Buenos Aires, Argentina.

ABSTRACT To study the process of regeneration and its effect on the morphology of the inhalant siphon of a tellineacean bivalve, *Donax denticulatus*, individuals were artificially amputated and placed in aquaria under laboratory conditions, in the absence of predators. After amputation, two individuals were sacrificed twice a day (9 AM and 5 PM) during a 9- day period, and their siphons were examined in the microscope. Within the first 24 hours after amputation, the process was slow, and only the rudiments of the primary tentacles were visible. However, between the second and the third day a fast transformation of the siphon was observed, with regeneration of secondary and tertiary tentacles. The siphon was fully regenerated on the fifth day after amputation. One month later, the siphon showed the same aspect than at the fifth day. Despite the delay observed in the regeneration of the siphon within the first 24 hours after amputation, probably due to physiologic constraints associated with the lack of well developed tentacles, regeneration showed to be a fast process. Development of primary and secondary tentacles must therefore play an important role in providing basic functionality to the siphon, as well as to the whole organism.

KEY WORDS: siphon, regeneration, *Donax denticulatus*, tellineacean bivalve, fish cropping

INTRODUCTION

To maintain their basic physiologic functions (i.e., feeding, defecation, and gas exchange), infaunal organisms must establish contact with the sediment-water interface. Soft bodied species often achieve this interaction by exposing parts of their bodies outside the sediment in the form of specialize structures such as ramified tentacles (e.g., polychaetes) or siphons (e.g., bivalves) that maximize the area of contact with the water column, thus optimizing the interaction with environment (McLachlan et al. 1995). However, the exposure of such structures can enhance the risk for predation, making infaunal organisms more vulnerable to tissue browsing by benthic feeders such as, crab and shrimp (Kamermans & Huitema 1994), pelagic juvenile flatfish (Trevallion et al. 1970), and birds (Zwarts 1986). Siphon cropping has been reported for several bivalve species (Trevallion 1971, Hodgson 1982, Peterson & Quammen 1982, Zwarts 1986). After amputation, siphons can regenerate providing a renewable source of secondary production that has been shown to maintain higher trophic levels (Penchaszadeh 1983, Pekkarinen 1984, Riera 1995, Luzzatto & Penchaszadeh 2001). However regeneration occurs at the cost of growth and reproduction, thus affecting the fitness of these organisms (Geller 1990, Peterson & Quammen 1982, Kamermans & Huitema 1994, Irlandi & Mehlich 1996).

The bivalve *Donax denticulatus* Linnaeus, 1758, locally known as “chipi-chipi” occurs intertidally on sandy beaches throughout the Caribbean all year long (Wade 1969). In Venezuela, it is commonly found in the intertidal zone of fine and medium grain dissipative sandy beaches in the central west coast, together with the

bivalves *Donax striatus* and *Tivela mactroides* (De Mahieu & Gamba 1980, Penchaszadeh 1983, Penchaszadeh et al. 2000). *D. denticulatus* is an important species in the food chain as a primary consumer of phytoplankton and detritus and is also eaten by a wide range of predators including the fishes *Menticirrhus littoralis*, *Conodon nobilis*, *Trachinotus carolinus*, *Trachinotus goodei* and *Unbrina coroides*, and the ghost crab *Ocypode verreauxi* (Penchaszadeh 1983, Riera 1995).

The siphons of this species are both flexible and extensible as are those of *Donax hanleyanus* Philippi 1847 (Luzzatto & Penchaszadeh, 2001) and other tellineacean bivalves. They are comprised of 6 primaries, 6 secondaries, 12 tertiaries, and 24 quaternaries tentacles (Wade 1969). When buried, the siphons of *D. denticulatus* extend through the sediment into the water column and look like a white crown from above. This highly ramified structure prevent large particules (>250 µm) from entering into the stomach (Wade 1969).

In this study, we followed a detailed examination of artificially amputated siphon regeneration of *D. denticulatus*, to establish time of regeneration and the effects of amputation on the morphology of siphons.

MATERIALS AND METHODS

Individuals of *D. denticulatus* were collected at the intertidal zone of Tucacas beach located in the central west coast of Venezuela (10°45'55"N, 68°19'24"W). Organisms with a shell length of 1.5–2.0 cm were collected to ensure that all were already sexually developed. They were transported to the laboratory in an icebox with wet sand. In the laboratory, 2 groups consisting of 50 animals each (control and experimental groups) were placed separately in aquaria with aerated seawater at 35‰, 23 °C and a layer of sand deep enough for burrowing. They were fed with Advanced Invertebrate Formula (Marine Enterprises, Inc.) once a day. The

*Corresponding author (Venezuela). E-mail: pmilos@usb.ve

*Corresponding author (Argentina). E-mail: pablop@mail.retina.ar

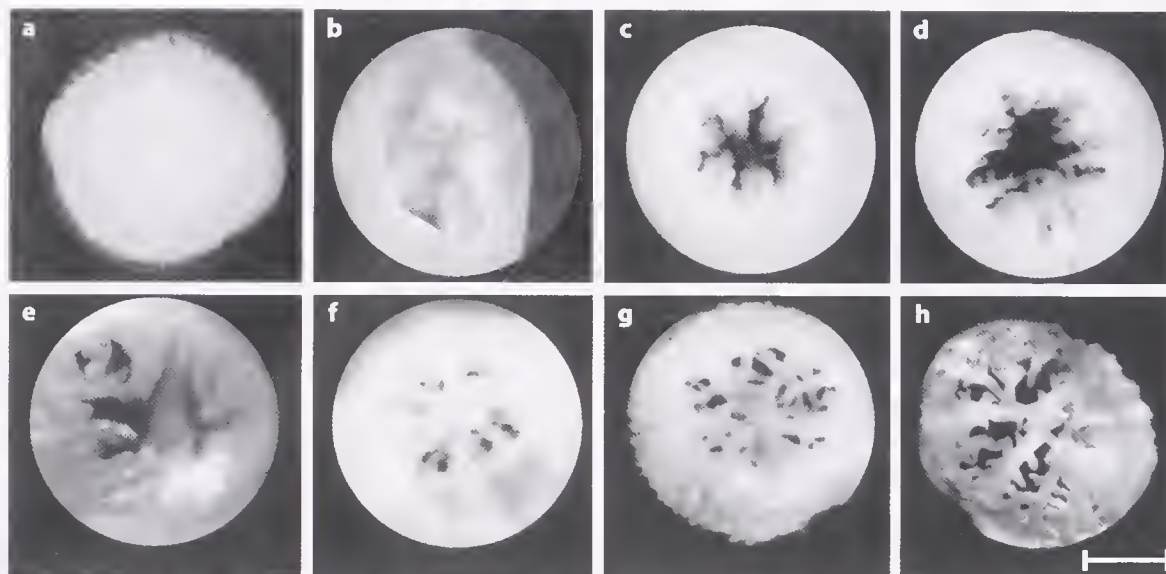


Figure 1. Sequence of inhalant siphon regeneration of *Donax denticulatus*, before amputation (a), 5 h after amputation (b), 24 h after amputation (c), 30 h after amputation (d), 54 h after amputation (e), 72 h after amputation (f), 4 days after amputation (g), 5 days after amputation (h). Scale bar: 0.5 mm.

animals were maintained for acclimation under these conditions for 5 days.

After this period, the experimental organisms were placed in Petri dishes with seawater. Once the siphons were extended, an anesthetic solution of magnesium chloride 7.5% in seawater was slowly added, and then the petri dish was placed in the refrigerator at 4 °C for 20 min. After this treatment, the siphons are completely elongated and do not retract in the shell, so we proceeded to cut the siphon tip with dissecting scissors. The tips were fixed in a glutamine-acetate buffer containing 6% formalin (Miloslavich & Penchaszadeh, 1997) and kept in the refrigerator. The animals were left to recover in their aquaria and then sacrificed in groups of 2 individuals twice a day (9 AM and 5 PM) during a 10-day period and then at day 30, cutting and fixing the tips by the described procedure. Observations of the fixed tips were done with a stereoscopic microscope. Also, we observed the siphons of the recovering animals to corroborate activity and functionality.

RESULTS

The regeneration sequence is shown in Figure 1. In the anesthetized individuals, the siphon slowly retracted into the shell after the cut and remained retracted for about 30 minutes. After this time, the siphon extended and started to function creating inhalant currents but unable to select or filter particles; some siphons were active up to 5 hours after the cut, and were able to select particles 72 hours after amputation. The siphon showed full functionality on the 5th day. Regarding the regeneration sequence, the 1st day (24 hours after the cut) the process was slow and only the rudiments of the primary tentacles were visible (see Figs. 1c). Within the 2nd and the 3rd day a fast transformation of the siphon was observed, with the regeneration of secondary tentacles (see Fig. 1d to f). The regeneration process was completed within the 5th day, however the regenerated siphon showed less ramification than the unamputated one (see Fig. 1h). A schematic sequence of regeneration is

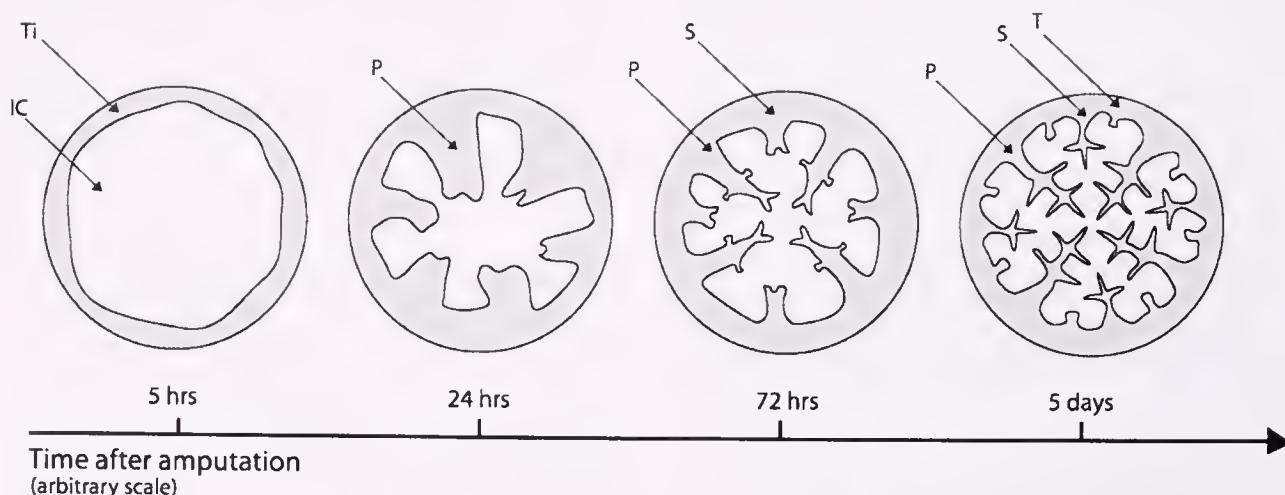


Figure 2. Schematic sequence of inhalant siphon regeneration 5 h after amputation, 24 h after amputation, 72 h after amputation, and 5 days after amputation. P, primary tentacles; S, secondary tentacles; T, tertiary tentacles; Ti, siphon tissue; I, internal cavity.

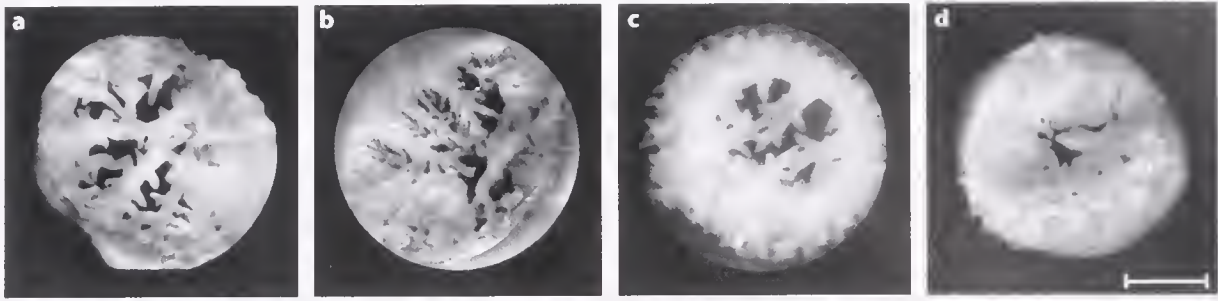


Figure 3. Regenerated siphon 5 days after amputation (a), 7 days after amputation (b), 9 days after amputation (c), and 30 days after amputation (d). Scale bar: 0.5 mm.

shown in Figure 2, highlighting the regeneration process of primary, secondary, and tertiary tentacles.

Between the 5th and the 9th day no significant transformations were detected (Fig. 3a to c). One month later, the siphon had the same as the 5th day (see Fig. 3d). At the end of experiments, all regenerated siphons were less ramified than unamputated ones, even 1 month after amputation.

DISCUSSION

As noted earlier, siphons are responsible for important activities such as feeding and defecation, therefore its ablation should have direct impact on these activities. Wade (1969) noted that after amputation, particles as large as 700 μm were found in the mantle cavity of *D. denticulatus*, forcing the organism to invest effort in extracting these particules out of the mantle, and temporarily suspending feeding activity. Additionally, regeneration itself is an energy consuming process. Artificially amputated individuals of *Donax serra* showed a decrease in growth rates when compared with nonamputated individuals (Hodgson 1982). Pekkarinen (1984) also registered significant growth rates differences in individuals of *Macoma balthica*, a tellinacean bivalve, when inhalant siphon was removed. The slow process of regeneration observed within the first 24 hours after amputation in our experiment, are probably due to the malfunctioning of the siphon, in the absence of developed tentacles, therefore limiting the food and oxygen intake of amputated individuals. Additionally, Pekkarinen (1984) observed that just after amputation, siphon muscles contract to close the wound, preventing the loss of hemolymph, but also preventing the water to flow through the siphons.

Luzzatto and Penchaszadeh (2001) studied changes in regen-

eration rates of inhalant siphon of *Donax hanleyanus*, and showed that this process had a sigmoidal behavior. Within the first hours, regeneration rate was slow, but then increased exponentially between the 1st and the 3rd day after amputation, until the secondary tentacles were developed. After that, regeneration rate slowed until the siphon became fully active in selecting particules (5 days after amputation). Siphons of *D. denticulatus* also showed fast regeneration rate between 24 and 72 hours after amputation (see Fig. 1d–f), with a full development of primary, secondary, and tertiary tentacles on the 5th day after amputation (see Fig. 1h). Pekkarinen (1984) reported full regeneration after 7 days for *M. balthica*.

Close observation of regenerating siphons allowed us to conclude that the highest regeneration occurred at the beginning of the process, with a short lag-time period of 24 hours, in which functionality was reestablished. The most notorious morphologic transformations were detected within the first 72 hours of regeneration, with fast regeneration of primary and secondary tentacles, whereas the regeneration of tertiary tentacles extended for a longer period of time (5 days). Therefore, primary and secondary tentacles must play an important role in providing basic functionality to the siphon, as well as to the whole organism.

ACKNOWLEDGMENTS

We are indebted to the students of the Marine Biology course of the Universidad Simón Bolívar who helped collecting the animals at Tucacas beach. This work was partially supported by a Decanato de investigación y Desarrollo (USB) grant to the Grupo de Ciencias Marinas (G-003) and by cooperative program Venezuela-Argentina funded by Fundación Antorchas.

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FIELD AND LABORATORY OBSERVATIONS OF THE MASS MORTALITY OF THE YELLOW CLAM *MESODESMA MACTROIDES* IN SOUTH AMERICA: THE CASE OF ISLA DEL JABALÍ, ARGENTINA

SANDRA FIORI,* VÍCTOR M. VIDAL-MARTÍNEZ,² RAÚL SIMÁ-ÁLVAREZ,² ROSSANNA RODRÍGUEZ-CANUL,² MA. LEOPOLDINA AGUIRRE-MACEDO² AND OMAR DEFEÓ^{2*}

¹Universidad Nacional del Sur San Juan 670, 8000 Bahía Blanca, Buenos Aires, Argentina;

²CINVESTAV Unidad Mérida, A.P. 73 Cordemex, 97310 Mérida, Yucatán, México

ABSTRACT Since the mid-1990s, the yellow clam *Mesodesma mactroides* (Deshayes, 1854) has experienced mass mortalities throughout its biogeographic range (23 °S to 41°S) along Atlantic exposed sandy beaches of South America. However, there is no information about the potential causes underlying these large-scale events. In the summer of 2002, a sudden massive mortality almost decimated the yellow clam population located to the south of Isla del Jabalí (Argentina), at the southern edge of its geographical range. Field observations showed a drastic decrease in abundance from 2000 to 2002, without recovering since then. Mortality sequentially occurred at the beach in a north-south direction, following the same trend as in the large-scale event. Significant differences in individual size were found between dead and live clams with the largest individuals of the population being the most affected. Histologic analysis revealed the presence of a meront stage of an unidentified coccidian parasite in the epithelium of the middle intestine of 3 of the 14 clams examined. Necrosis was found in gills and stomach in 13 of the 14 clams examined. The results suggest that these parasites could play a role in the massive mortality events of the yellow clam populations all along South American sandy beaches since 1993. These findings provide an alternative explanation to the widely held notion that mass mortalities in sandy beach macrofauna are due to harmful algae blooms.

KEY WORDS: mass mortalities, bivalves, *Mesodesma mactroides*, coccidian parasite, sandy beaches, Argentina

INTRODUCTION

The yellow clam *Mesodesma mactroides* is an intertidal bivalve distributed along the warm-temperate Atlantic coast of South America, from Sao Paulo State, Brazil (24 °S) to the south of the Buenos Aires province, Argentina (41°S; Fig. 1a). Yellow clam populations prosper primarily in the intertidal zone of microtidal dissipative beaches with gentle slope, fine sand, heavy wave action, and a wide surf zone often characterized by high primary production by surf diatoms (Defeo & Scarabino 1990). The yellow clam has supported recreational and artisanal fisheries of high socio-economic importance (Defeo et al. 1993, Castilla & Defeo 2001).

Since 1993, episodic mass mortalities have decimated yellow clam populations throughout its entire geographic range. These events occurred mainly between late spring and early summer, following a sequential north-south direction (see Fig. 1a). Mortalities occurred first in Brazil in March 1993 (Odebrecht et al. 1995), then in Uruguay in December 1994 (Méndez 1995) and last in Argentina from September to November 1995 (Fiori 1996, Fiori & Cazzaniga 1999). These mass mortalities have prevented the rehabilitation of yellow clam populations throughout its range (Fiori 2002, Defeo 2003). These large-scale events have not been successfully explained, mainly because of the lack of sampling opportunities concurrent with the occurrence of these sudden phenomena. Odebrecht et al. (1995) conjectured that high abundance of dinoflagellates was a source of yellow clam mortality in Brazil, but when the mass mortality occurred in Uruguay, bioassays indicated that paralytic shellfish toxins were not present (Méndez 1995). The same held true for Argentinean coasts, where metal contamination, phytotoxins, abnormal phytoplankton composition,

and protozoan tissue-parasites were discarded as causative factors (Fiori & Cazzaniga 1999).

In January 2002 (austral summer), a mass mortality occurred in the southernmost and isolated yellow clam population located at the south of Isla del Jabalí, Argentina. During this event, living organisms were recovered for histologic study. Here we describe the mass mortality event at Isla del Jabalí. First, we quantify the magnitude of the event in terms of population density and structure. Second, we perform a histologic examination of the visceral mass of clams, including gills, digestive gland and stomach. Finally, we discuss the large-scale implications of these findings.

MATERIALS AND METHODS

The study area located to the south of Isla del Jabalí (Argentina: 40°33'S; 62°14'W) is a continuous sandy beach, ca. 15 km long, with gentle slope ($1.64 \pm 1.16^\circ$), fine sand (mean grain size = 0.20 ± 0.91 mm), strong wave action and high salinity (34.3 ± 0.17 ppm) (Fiori 2002). The mean air temperature fluctuates between 6 °C (winter) and 19 °C (summer). During the mortality event, a stratified random sampling was carried out in 3 beach sites separated by 4 km and located in the north-south direction, thereafter mentioned as sites 1, 2 and 3 (see Fig. 1b). A total of 18 random parcels were located in the mesolittoral area inhabited by the yellow clam, using 50 × 50 cm frames and digging up to a depth of 50 cm. The sediment from each parcel was sieved through a 1-cm mesh and all clams retained were counted, measured with calipers (0.1-mm precision) and discriminated as dead or alive. All clams collected were used to estimate the length frequency distribution (LFD) of the population discriminated by site and clam status: (1) dead clams, in life position into the sediment with the foot and siphons everted and the shell paired open, or with decomposed flesh; (2) alive clams, with closed shell and ability to burrow themselves in the substrate. We did not consider empty valves placed on the sand because we cannot attribute incontestably if

*Corresponding author. E-mail: odefeo@mda.cinvestav.mx



Figure 1. (a) Geographic distribution of the yellow clam *Mesodesma mactroides* along Atlantic sandy beaches of South America, highlighting the locations and years when mass mortality events occurred; (b) Isla del Jabali, with the three beach sites sampled in February 2002.

these valves came from the mortality event. Two-way ANOVAs were used to test for differences between density and clam length, with status and beach site as main factors. A simple length-based survival ratio SR was applied as $SR = 1 - D_i/(D_i + L_i)$, where D is the number of dead clams and L is the number of live clams for each length class i . The analysis was compared with density estimates obtained in summer of 2000, following the same stratified random sampling detailed above.

A total of 14 clams collected in sites 2 and 3 (we did not find clams in site 1) were fixed after shucked in 10% buffered formaldehyde in seawater immediately after collection and transported to CINVESTAV-Merida (Mexico) for histologic examination. After arrival, formaldehyde was washed out and replaced with Davison's AFA solution for 4 days. Then, a section of the visceral mass of each preserved clam that contained gills, digestive gland and stomach was embedded in paraffin and cut at 5 μ m thickness. The tissue sections were affixed to glass slides and stained with hematoxylin and eosin (Howard & Smith 1983). A frequently causal agent of mass mortality in oysters, clams, cockles, scallops, and abalones documented worldwide is the Apicomplexan protozoan *Perkinsus* spp. (Bower et al. 1994). Unfortunately, thioglycollate fluid or other culture media to determine the presence of these Apicomplexan parasites were not available during the mortality event. In this case, we had to rely on the histologic findings. Samples of clam tissue embedded in paraffin were also sent to Dr. Eugene Bureson (Virginia Institute of Marine Science) for detecting *Perkinsus* spp. using a genus-specific DNA probe. A *Perkinsus marinus*-specific PCR test based on the primers of Robledo et al. (1998) was also undertaken at CINVESTAV Merida. Finally, digital photographs of the parasites were sent to other experts in coccidian parasites (Dr. Donald Duszynsky, University of New Mexico, USA; Dr. Iva Dykova, Institute of Parasitology of the Academy of Sciences of the Czech Republic) for identification.

RESULTS

Dramatic declines in abundance of yellow clam were observed in two of the three sites sampled at Isla del Jabali. About 15 km of the beach was covered by an accumulation of empty shell, particularly on the supralittoral zone (Fig. 2). The moribund yellow clam remained buried into the sand, even though they weakly retracted the siphons after a mechanical stimulus. Table 1 shows the clams density before and after the mass mortality event. Density significantly differed between sites ($F_{2,30} = 18.31$; $P < 0.001$), but did not differ between clam status ($F_{1,30} = 0.77$; $P < 0.973$). The 'site \times status' interaction factor was significant ($F_{2,30} = 3.95$; $P = 0.03$), as a result of the contrasting patterns in clam status between sites 2 and 3 (LSD test, $P < 0.01$; see Fig. 3a).

In site 2, individual size of live clams were in the range 45–67 mm (mean \pm SD: 61.8 ± 5.9 mm), whereas dead clams varied between 51 and 76 mm (65.3 ± 4.1 mm). In site 3, dead yellow clam varied between 40 and 77 mm (mean \pm SD: 65.2 ± 8.3 mm), whereas live clams were in the range of 54–80 mm (67.3 ± 6.1 mm). The survival ratio SR markedly differed between sites (see Fig. 3b): in site 2, SR exponentially decreased ($R^2 = 0.69$; $P < 0.01$) towards larger clam sizes, with 0% survival at sizes >70 mm; in site 3, size-specific SR were significantly higher than in site 2, and linearly decreased ($R^2 = 0.40$; $P = 0.02$) towards oldest length classes, reaching values as high as 67% at 76 mm. The two-way ANOVA revealed significant differences in mean size between beach sites ($F_{1,272} = 5.74$; $P < 0.017$) and clam status ($F_{1,272} = 5.06$; $P < 0.025$), while the 'site \times status' interaction factor was not ($F_{1,272} = 0.53$; $P = 0.468$). This means independence of the effect of "site" on the level of the factor "status", reflected in highest sizes at the northern site and for dead clams (LSD test; $P < 0.01$).

Eleven of the 14 (78%) clams examined presented necrosis in the stomach (Fig. 4a), which was characterized by necrotic hemocytes and pycnotic cells (see Fig. 4a). Thirteen of the 14 (93%) clams also presented necrosis in gills (see Fig. 4b). The ciliate *Trichodina* sp. was found in one of the 14 (7%) clams. Samples did not react with the *Perkinsus* genus-specific DNA probe, nor were positive for the PCR test for *Perkinsus marinus* either. The parasite in Figure 4c was a meront stage of an unidentified coccidia 21–16 μ m long and 13–17 μ m wide ($n = 10$), with

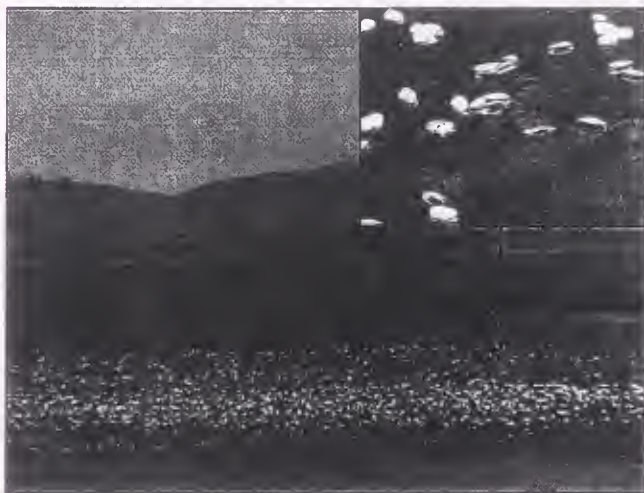


Figure 2. Dead yellow clams, *Mesodesma mactroides*, during the massive mortality event in February 2002 at Isla del Jabali.

TABLE 1.

Mean (\pm SD) density of *Mesodesma mactroides* (ind/m²) per site at Isla del Jabalí beach.

SITE	2000	2002
1	189.33 \pm 140.93	0
2	68.00 \pm 76.00	12.00 \pm 9.12
3	94.67 \pm 18.90	110.00 \pm 90.54
Total	102.77 \pm 89.79	40.67 \pm 70.75

16–21 merozoites per meront. The merozoites were elongated with 4–5 μ m long and 2–3 μ m wide ($n = 10$). This parasite was present in 3 of the 14 (21%) clams examined. There was necrosis of the tubules of digestive diverticula in the infected host (see Fig. 4d).

DISCUSSION

We provide the first observations for the mass mortality that decimated the yellow clam population at the southernmost edge of its range. Sites 1 and 2 showed a dramatic decrease in density, whereas site 3, situated southwards, appeared to be unaffected in the short term during the mortality event. This gives support to the directional event from north to south, as occurred on a biogeographic scale. However, it is worth mentioning that, after that date, all clams in the three sites died and up to now (June 2004) the recovery of the population has been negligible (Fiori, personal observation). Analysis of the LFDs and the size-based *SR* showed

that larger clams were more susceptible than smaller ones. Survival significantly decreased towards larger sizes, and this was consistent for the two main beach sites analyzed, even though the declining ratio was exponential in site 2 and linear in site 3, suggesting again a north-south direction of the mortality event.

The histologic analysis revealed the presence of gill, stomach, and intestine necrosis. It is difficult to link this tissue damage to the coccidian parasite found, because it was present only in 3 of the 14 clams examined. However, it is not possible to discard the possibility either, because the collected clams were survivors of the massive mortality. In these circumstances, it would be reasonable to expect that collected specimens should have low infection levels. In addition, Cremonte and Figueras (2003) reported, for the first time for this species, coccidian and gregarines in a sample of yellow clam from Isla del Jabalí, which was taken 2 years before the mass mortality. Coccidians were found in 93% of the 32 clams processed. Furthermore, several authors have found that mass mortalities of other mollusks (scallops) in the Atlantic coast of America and Spain have been related to coccidian parasites, especially those of the genera *Pseudoklossia* and *Margolisella*. These parasites produced extensive destruction of kidney and digestive gland of their hosts (Leibovitz et al. 1984, Desser & Bower 1997, Carballal et al. 2001). Unfortunately, it was not possible to examine kidney because this tissue did not appear in the fixed material. However, it is not possible to attribute the necrosis to bad fixation of the biologic material because all clams were properly shucked and fixed. Clearly, more research is needed to determine the relationship between this coccidian parasite and the tissue damage observed.

The mass mortality at Isla del Jabalí occurred in summer, in agreement with the other yellow clam mortality events that have occurred throughout the species range (Fiori & Cazzaniga 1999). This indicates high vulnerability of the yellow clams during this season, concurrently with high temperature, salinity and species density (Fiori 2002). A possible explanation for this pattern could be an increase in the vulnerability of the parasitized clams with respect to the unparasitized ones. For example, *Perkinsus*-associated mass mortalities have been observed with high values of temperature, salinity, and species density (Choi et al. 2002). Mass mortalities tend to occur in summer (Chu et al. 1996, Choi et al. 2002) or even as a result of increasing winter water temperatures (Cook et al. 1998). However, to the best of our knowledge, there are not published studies on the seasonal variation of the infection parameters (prevalence, mean intensity) or mortality produced by coccidian parasites of bivalves, and its association with environmental factors such as temperature or salinity remains unknown.

One of the most important characteristics of the mass mortalities affecting the yellow clam throughout South America is its strong host specificity, as other members of the macrofauna, including the very abundant sympatric suspension feeders in Uruguay and Brazil, *Donax hanleyanus* (Defeo & de Alava 1995) and *Emerita brasiliensis* (Defeo & Cardoso 2002) were not affected (Defeo 2003). As transplanting of clams has not been performed, it seems that the mortality agent is transported by littoral currents, suggesting a strong dispersal capability and tolerance to surpass zoogeographic barriers like the estuarine environments Rio de la Plata, Bahía Blanca, and Bahía Anegada, which present highly variable physico-chemical conditions, especially in salinity.

Our results, even preliminary, provide the first potential explanation to the mass mortality event in this species. Further studies involving immunologic and molecular biology techniques are

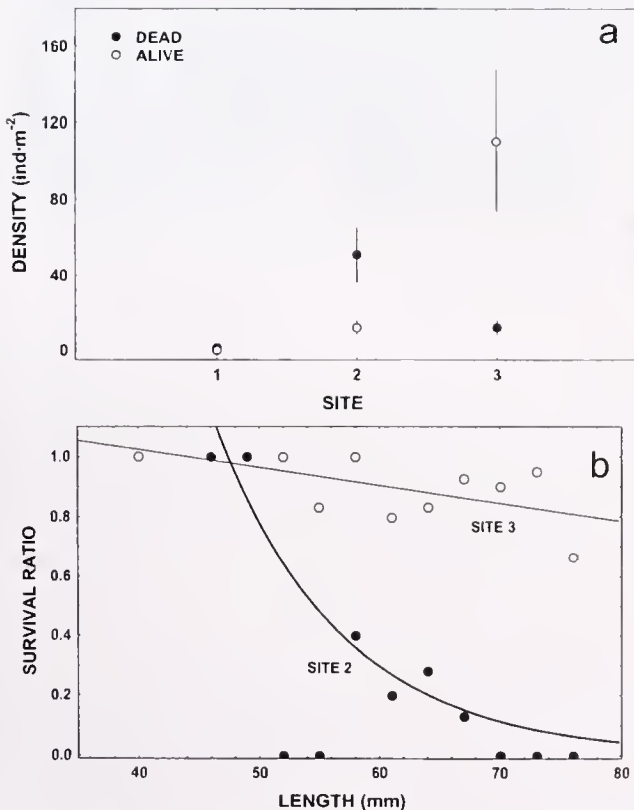


Figure 3. *Mesodesma mactroides* at Isla del Jabalí, summer 2002. (a) Mean (\pm SE) density during the mass mortality event, discriminated by site and clam status; (b) variations in the length-based survival ratio for sites 2 and 3, with the corresponding functions fitted.

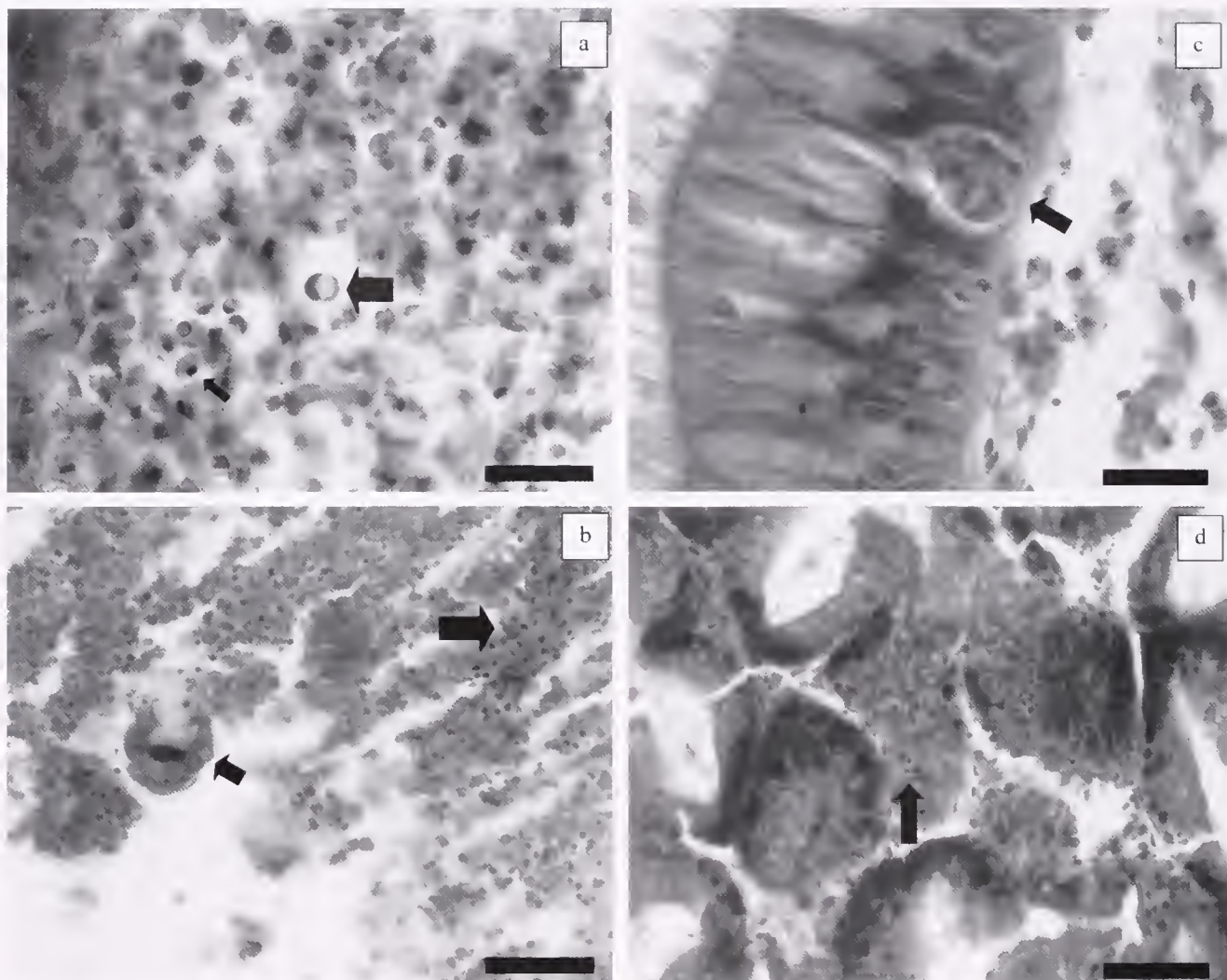


Figure 4. Histologic section showing (a) extensive necrosis in the epithelium of the stomach, a necrotic hemocyte (large arrow), and a cell with pyknotic nucleus (small arrow); (b) necrosis in gills lamellae (large arrow) and *Trichodina* sp. (small arrow); (c) section of the epithelium of the middle intestine showing a meront of an unidentified coccidian parasite (arrow); and (d) necrosis of the tubules of digestive diverticula (arrow). H & E stain. Scale bar = 15 μ m.

needed to provide conclusive results about the role of this parasite in the massive mortality of this endangered species (Fiori & Cazaniga 1999).

ACKNOWLEDGMENTS

This paper was written during the post-doctoral stage of Sandra Fiori at CINVESTAV-Merida, with a fellowship granted by

CONACYT and Secretaría de Relaciones Exteriores (Mexico). The authors thank Susana Castelnuovo and Alberto Alzugaray for field support. We are indebted to Prof. Eugene Bureson (VIMS, USA), Dr. Donald Duszynski (University of New Mexico, USA) and Dr. Iva Dykova (Institute of Parasitology of the Academy of Sciences of the Czech Republic) for their help in the identification of the coccidian parasite.

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IMPACT OF GREEN CRAB (*CARCINUS MAENAS* L.) PREDATION ON A POPULATION OF SOFT-SHELL CLAMS (*MYA ARENARIA* L.) IN THE SOUTHERN GULF OF ST. LAWRENCE

TREVOR FLOYD AND JIM WILLIAMS*

Department of Biology, St. Francis Xavier University, Antigonish, Nova Scotia, B2G 2W5, Canada

ABSTRACT A caging experiment was carried out on an estuarine mudflat in Pomquet Harbour, Nova Scotia, Canada from late May to early September 2001. Six replicate 0.83 m² cages were set up for each of the following treatments: low predator density cages with one green crab (*Carcinus maenas*), high predator density with five green crabs, enclosure cages with no crabs, and control cages with sections of the sides removed. In addition, six 0.83 m² sections of exposed mudflat served as further controls. Green crabs significantly reduced numbers of small (<17 mm) soft-shell clams (*Mya arenaria*), removing ca. 80% of the small clams within low and high predator cages. There was no significant difference in large (>17 mm) soft-shell clam densities for any of the treatments. Green crabs consumed small clams at a minimum rate of 3.1 clams/crab/day and a maximum rate of 21.8/clams/crab/day. Predation intensity did not vary with density of crabs within cages.

KEY WORDS: green crab, *Carcinus maenas*, invasive, soft-shell clam, *Mya arenaria*, predation, caging experiment

INTRODUCTION

The establishment of an invasive or non-indigenous species can result in significant ecological changes in the receiving environment. The green crab, *Carcinus maenas* (Linnaeus), is an example of a very successful marine invasive species. After establishment in a new environment, green crabs have been shown to effect changes at the individual, population and community levels (Hughes & Elner 1979, Grosholz et al. 2000, Trussell & Smith 2000, McDonald et al. 2001).

Although green crabs directly and indirectly affect many benthic organisms, bivalves are usually the preferred prey (Ropes 1968, Elner 1981, Grosholz & Ruiz 1995) and exhibit dramatic post-invasion changes. Grosholz et al. (2000) found that invasive green crabs exert predatory control on the native clams *Nutricula tantilla* (Gould) and *Nutricula confusa* (Gray), population levels of these clams declined drastically within 3 years of the arrival of green crabs, and have shown no sign of recovery since the invasion. In Tasmania, Walton et al. (2002) used both field observations and manipulative field experiments to investigate green crab predation on the venerid clam *Katelysia scalarina* (Lamarck). They found that green crabs exerted considerable predatory pressure on smaller clams (<13 mm shell length). On the east coast of North America, declines in population sizes of the soft-shell clam (*Mya arenaria* Linnaeus) have been linked to the arrival of the green crab (Glude 1955).

It has been established that soft-shell clams are a significant prey item for green crab. Glude (1955) found excellent survival and growth in areas where green crabs were excluded by fencing or screening. Ropes (1968) examined approximately 4000 green crab stomachs, and concluded that soft-shell clams were an important component of the crab's diet in Massachusetts. He also reported that the frequency of *M. arenaria* remains in green crab stomachs was highest during September to November, and suggested that this was due to the abundance of juvenile clams in the fall. In a similar study from Port Hebert, Nova Scotia, Elner (1981) also found that soft-shell clams were an important prey item for green crabs. However, in contrast to the New England study, Elner (1981) reported significant predation on soft-shell clams in May and August.

In summary, correlations have been demonstrated between green crab arrival and declines in abundance of soft-shell clams. Furthermore, gut content studies have shown that soft-shell clams are a significant prey item for green crabs. We used an *in situ* caging approach to investigate the timing, nature, and extent of green crab predation on a population of soft-shell clams. Specifically, we wanted to: (1) experimentally determine the extent of green crab predation on soft-shell clams during the summer; (2) determine if green crab predation on soft-shell clams was size selective; and (3) determine if there was a relationship between green crab density and predation rate.

METHODS

Pomquet Harbour, Nova Scotia, is a shallow estuary (tidal amplitude of approximately 1 m) that is connected by a narrow channel with St. Georges Bay (Fig. 1). The study site was a 7 × 11 m section of a lower intertidal flat of slightly gravelly sand located near the mouth of the Pomquet River (45°36.19'N, and 61°36.19'W). The surrounding terrestrial vegetation was predominantly *Spartina alterniflora* (Loisel) with patches of *Solidago sempervirens* (L.), *Convolvulus arvensis* (L.), *Festuca pratensis* (Hudson), and *Rosa virginiana* (Mill.). Salinity and temperature at the site were measured from high tide to low tide for one spring tide on August 5, 2001. The surface salinity ranged from 26.0‰ to 23.2‰ and the bottom salinity ranged from 29.0‰ to 25.1‰ during the ebb. The surface temperature ranged from 21.2°C to 26.3°C and the bottom temperature ranged from 21.8°C to 26.3°C. The water depth in the middle of the study site was approximately 35 cm at high tide, and the mudflat was approximately 20 cm above sea level at low tide.

On May 22, 2001, limited preliminary samples were collected to identify the macrofauna, with emphasis on *M. arenaria*. The sampling was limited partly to minimize disturbance on the relatively small clam flat, and partly because the design was not based on comparisons of pre- and post-experimental conditions. Six core samples (10 cm diameter, 20 cm depth) were taken from randomly selected areas around the proposed location for the cage matrix. The 6 core samples were washed through a series of graded sieves to a bottom mesh size of 0.5 mm, and all retained organisms were removed and preserved. This mesh size has been shown to yield close to 100% of the macrofaunal species and over 95% of the biomass (Reish 1959). Also, sediment from a 1 m² section of the

*Corresponding author. E-mail: jwilliam@stfx.ca

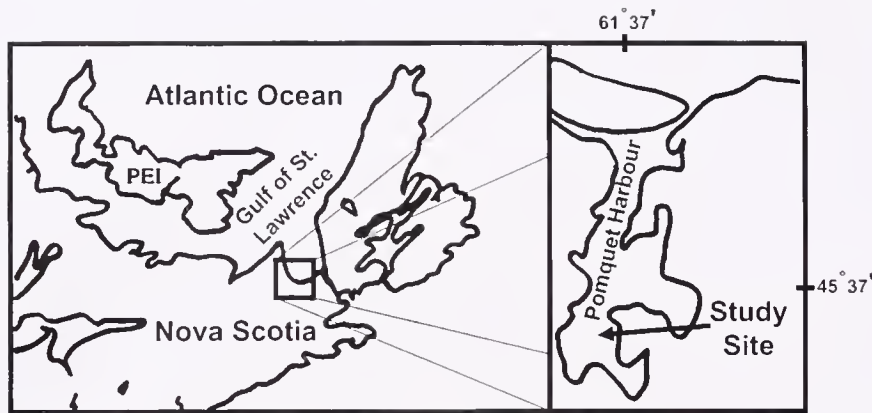


Figure 1. Map of study site in north eastern Nova Scotia, Canada. Expanded panel indicates cage experiment site in Pomquet Harbour.

site was removed to a depth of 20 cm and passed through a 1.27 cm sieve to collect the large *M. arenaria* (clams >17 mm). We designated clams larger than 17 mm as large soft-shell clams for the purpose of this experiment. This size represents the maximum observed size of that year's set of small, recently settled clams.

Cages were $0.91 \times 0.91 \times 0.30$ m high, and were constructed of plastic-coated wire, with a square mesh opening of 1×1 cm. Treatments were as follows: 6 enclosure cages (E), with no predators added, 6 control cages (C1) with 20×70 cm portions of each side removed allowing for unrestricted predator movement, 6 undisturbed mudflat controls that were outside but adjacent to the cage matrix (C2), 6 low-predator-density cages (D1) with 1 crab added, and 6 high-predator-density cages (D2) with 5 crabs added. The low predator densities of 1.2 crabs/m² reflects mark and recapture data acquired the previous year in a similar habitat in an adjacent harbor (Campbell 2000). The high predator density of 6.1 crabs/m² reflects green crab densities estimated by Young et al. (1999) for a New England salt marsh tidal creek.

To reduce the potential impact of any environmental heterogeneity of the mudflat, a randomized block design was used for location of cages. During a spring low tide on May 23, 2001, the cages were deployed in a 6×4 rectangular matrix, with the four treatments randomly assigned to a position in each row (= block), and 1 m between all the cages. The rows were oriented perpendicular to the gradual slope of the mudflat, resulting in approximately 10 cm height difference between the highest and lowest cages. The cages were pushed into the sediment to a depth of 20 cm, allowing a 10-cm space above the substratum. Green crabs trapped the previous day were added to the appropriate cages by cutting a small flap in the cage, adding crabs, and closing the flap with plastic cable ties. Only male crabs (53.5 ± 5.22 (SD) mm, ranging from 44 mm to 65 mm in CW) with complete pairs of chelipeds and sets of walking legs were used in the enclosures. The crabs in the D1 and D2 cages were checked every spring tide throughout the next 3 months by gently probing the mud with a wire probe. Following Gee et al. (1985), any crab mortality during the experiment was noted, but crabs were not replaced.

Between August 21 to 23 (approximately 3 mo after deployment), the cages were removed from the substratum. To test for cage-induced physical changes to the site, 4 random sediment samples were taken (5 cm deep, 2.5 cm diameter) from each cage area, as well as the 6 C2 plots sampled outside of the cage matrix. These samples were frozen and later dried at 70°C for 7 hours. A weighed subsample was heated in a 500°C muffle furnace for 7

hours and re-weighed to determine the percent organic content, which was compared among treatments using the Kruskal-Wallis non-parametric H-test. Following Folk (1974), subsamples of the remaining sediment were then dry sieved through a series of Wentworth graded sieves to determine the size distribution of the particles. These size distributions were then compared with check for cage effects.

Three large cores (10 cm diameter, 20 cm depth) were taken from randomly selected locations within each cage (Hall et al. 1990). To avoid edge effects, cores were never taken within 15 cm of the sides of the cages (Kent & Day 1983). The core samples were sieved to a bottom mesh size of 0.5 mm, and clams preserved and counted. After coring, all sediment inside the cages was excavated to a depth of 30 cm and sieved through a 1.27-cm coarse sieve to collect large soft-shell clams. Large clams were counted and a concentric ring analysis was used to determine age (Newcombe 1936). In addition to sampling of cage sites, the six C2 plots, which were chosen randomly on all sides of the cage matrix, were subjected to the same sampling regimen.

Core sample data was used to determine densities of small soft-shell clams (clams <17 mm in length). Since three cores were taken from each cage, the arithmetic means of the cores were used in the analysis to avoid problems of pseudoreplication (Gee et al. 1985). The Shapiro-Wilk method was used to test for the normality of the count data for both size classes of clams. Data for large and small soft-shell clams was normally distributed ($P = 0.13$ and 0.42 respectively). An ANOVA appropriate for a randomized block design and the Tukey multiple comparison test was used to test for significant difference among treatments. Initially we only included the 4 cage treatments E, D1, D2, and C2 in the analysis. Because no block effect was detected in the initial analysis ($P = 0.157$), a subsequent analysis was run with the additional data from the mudflat C2.

On May 30, 2002, the study site was again sampled to determine the fall/winter survival of the 2001 summer set of small soft-shell clams. Six random core samples were obtained adjacent to the study site and clams were removed, measured, and counted.

RESULTS

No noticeable scouring or deposition of sediment from water movement was observed in cages throughout the experiment. Sediment organic content averaged $3.30\% \pm 0.57$ (SD), with no significant difference among treatments ($\chi^2 = 2.612$, d.f. = 5, $P =$

0.625). Based on particle size distribution, the sediment for the study site can be classified (Folk 1974) as slightly gravelly sand. Particle distribution analysis showed no difference between treatments (Fig. 2). No long-term fouling by growing or drift algae occurred on the cages.

The core samples taken before the cages were deployed yielded 3 species of macrofauna: the bivalves, *M. arenaria* and *Macoma balthica* L., and the polychaete, *Hediste diversicolor* Muller. Soft-shell clams made up more than 95% of macrofaunal numbers and biomass. Large soft-shell clams had a pre-experiment density of 21/m². There were no small clams found at this time.

No significant difference was found among treatments for large soft-shell clams, both when only cage data were compared (Fig. 3, $P = 0.101$) and when undisturbed mudflat C2 data were included ($P = 0.134$). Large soft-shell clam densities ranged from 11 to 24 clams/m², and had a mean density of 19.2 ± 6.05 (SD) clams/m², (see Fig. 3). A significant difference was found among treatments for small soft-shell clams, when the cage data were compared, and when the undisturbed mudflat C2 was included (Fig. 4, $P < 0.001$ for both comparisons). Specifically, D1 and D2 enclosures had significantly fewer small clams than all other treatments but did not differ from each other. Small soft-shell clam densities ranged from 305/m² (D2 enclosure) to 1712 clams/m² (enclosure).

Some crab mortality occurred in enclosure cages during the last 3 weeks of the experiment, thereby making calculation of consumption rates difficult. Average estimated consumption rates were calculated using the initial and final crab densities. Two of six crabs in the D1 cages died during the last month, yielding a final D1 density of 0.80 crabs/m². In the D2 treatments, which started with 5 crabs in each cage, the number of surviving crabs were 0, 1, 2, 2, 3, and 3 crabs, yielding a final D2 density of 2.16 crabs/m².

Because no significant difference was detected among treatments for large soft-shell clams, the age analysis data were pooled to provide a picture of the age structure at the study site (Fig. 5). The 4-year and 5-year age classes dominated the sampled soft-shell clams on the study site, with very few clams aged 1, 2, and 3 years in the population. The spring 2002 sampling yielded 148 small soft-shell clams/m². This spring density was significantly

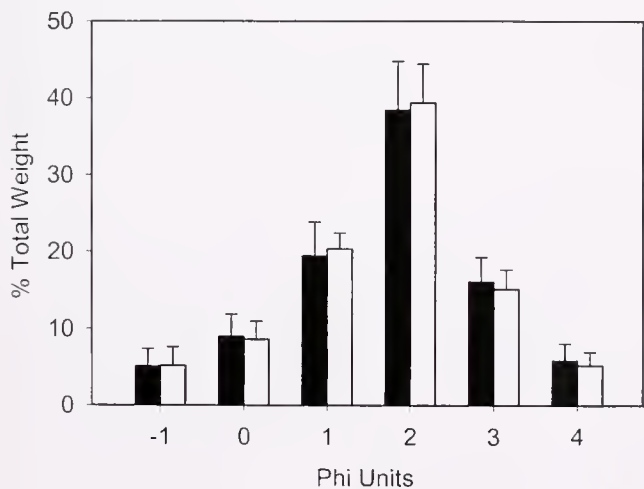


Figure 2. Histogram of particle size distribution (in Φ units) of sediment samples from enclosure cages (open bars) and uncaged mudflat (filled bars). Height of bar indicates means, error bars are 95% Confidence Intervals.

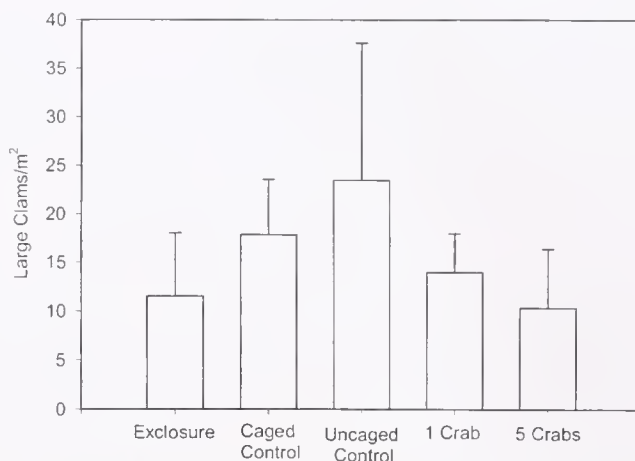


Figure 3. Densities (numbers of clams per square meter) of large soft-shell clams (>17 mm shell length) from experimental cage plots. Bars represent means, error bars represent standard deviations of means.

less than the late summer density of approximately 1500/m², and represents nearly 90% fall/winter mortality.

DISCUSSION

Small Soft-shell Clams

We found clear evidence of significant green crab predation on soft-shell clams with small clam densities within enclosures significantly lower than in all other treatments. This concurs with a summary of green crab feeding studies provided by Cohen et al. (1995), in which they report that green crabs select soft-shell clams less than 20 mm in length. Welch (1968) found that green crabs regularly destroyed sets of small clams, and Beal et al. (2001) states that losses of recently settled soft-shell clams coincided with the appearance of predators, including green crabs, within experimental units. Glude (1955) found a daily consumption rate of 15 small clams/crab in the laboratory. Our ability to calculate daily consumption rates is somewhat hampered by the crab mortality that occurred in the last 3 weeks of the study, and our inability to

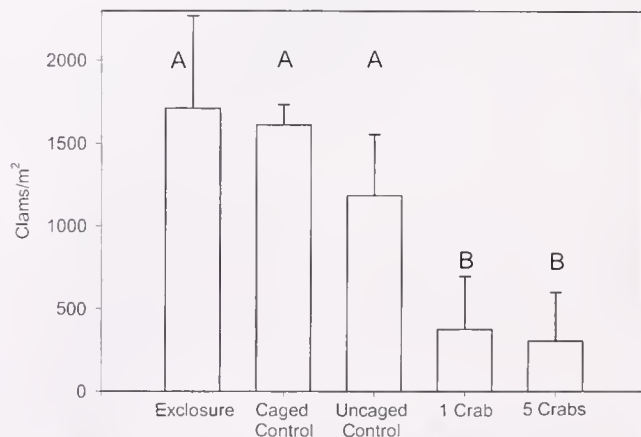


Figure 4. Densities (numbers of clams per square meter) of small soft-shell clams (<17 mm shell length) from experimental cage plots. Bars represent means, error bars represent standard deviations of means. Different upper-case letter above bars indicates a significant difference between means.

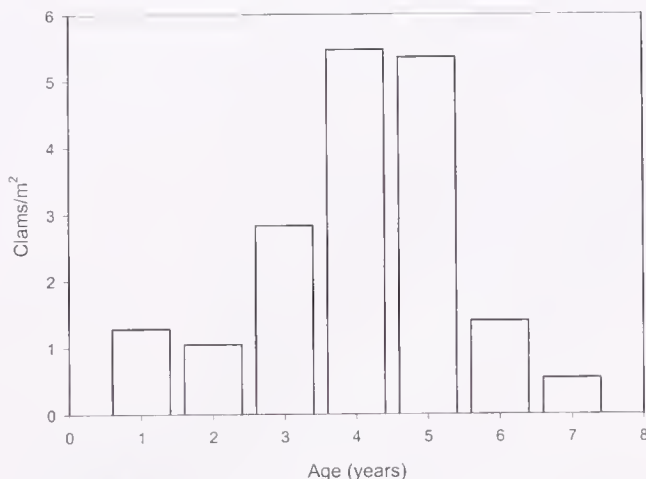


Figure 5. Observed densities of each age group of large (>17 mm shell length) soft-shell clams from the experimental cage site. Each bar represents the density of one age class, determined by the total counts from all 30 cage sites divided by the total surface area exhumed (approximately 25 square meters).

determine when clams were consumed. However, we can calculate a range by assuming either that all clams were consumed prior to crab mortality, or that all clams were consumed by the remaining crabs after the mortality occurred. Daily consumption rates were between 14.5 and 21.8 small clams/crab in the one crab treatments and 3.1 and 8.3 small clams/crab in the five crab treatments. These ranges are the first *in situ* estimates of green crab consumption rates on small soft-shell clams.

One of the objectives of this research is to assess the extent of summer predation on soft-shell clams. A comparison of clam densities among enclosure cages, control cages, and open mudflat samples provide estimates of overall predation, and also gives some clue as to the type of predator. The control cages were designed to exclude avian predators, but allow access by fish and invertebrates. The open mudflat sites were available to all predators. No statistically significant differences were detected between enclosure clam densities and densities from the two types of control samples. Even though our results demonstrate that green crabs are capable of harvesting a significant number of small soft-shell clams from the flats, this activity does not take place during the summer. Ropes (1968) found soft-shell clams occurred most frequently in green crab stomachs during September to November. As well, Glude (1955) found that strongly recruiting populations of small soft-shell clams had disappeared by the autumn where green crabs were present. These reports, as well as this study, are in contrast with Elner (1981), who found that *M. arenaria* were a large component of green crab diet in both May and August. The difference in the timing of crab predation on soft-shell clams in Elner's (1981) work may be due to much cooler summer water temperatures along the southeastern coast of Nova Scotia, as compared with the two New England studies, and our estuarine site in Pomquet Harbour. On August 5, maximum bottom temperatures at our study site were 26°C, and could well have exceeded 30°C in August. In the late fall, when the water temperatures are lower, green crabs may increase foraging on the clam flats. At this time, the crabs would also benefit from the increased size of young-of-the-year clams. Extensive pitting attributed to green crab foraging has been observed on other Pomquet Harbour mudflats during the late autumn and early winter (P. J. Williams, unpublished data).

To obtain an estimate of small clam mortality during the fall/winter period, we sampled the study site the following spring (2002) before the green crabs became active. We found that approximately 90% of the small soft-shell clams had been removed in the period from August 2001 to May 2002. Although we cannot partition the removal among migratory birds, fish, green crab, other invertebrates, or physical factors such as ice scour, the loss is comparable with the 80% mortality we observed within green crab enclosures.

There was no difference in small soft-shell clam abundance between the low density (1 crab) and the high density (5 crabs) crab enclosures. Crab mortality during the experiment may have compromised our ability to detect differences. In retrospect, an experiment with a shorter time frame would have been better to elucidate density-related predation rates. However, even green crab densities of 0.8/m² can result in effective removal of 80% of small clams.

Large Soft-shell Clams

In contrast with the small soft-shell clam results, we found negligible predation by green crab on large soft-shell clams. The apparent selection of small clams over large ones has been suggested for green crab feeding on soft-shell clams (Welch 1968), and indeed fits a general pattern for crustaceans feeding on molluscs (Smith et al. 1999). There are a number of factors that might have led to the size discrimination. Green crabs are both tactile and chemosensory hunters (Cohen et al. 1995), and probably detect clams by following plumes from exhalant siphons, and/or coming in contact with siphons or siphon holes while probing sediment with appendages (Dare & Edwards 1981). During this study, the densities of small clams were much higher than the large clams, and therefore green crabs would have a higher encounter rate with the smaller clams. Studies that have addressed crab predation on molluscs from an energy optimization viewpoint (Elner & Hughes 1978, Juanes 1987) suggest that thicker shells in larger bivalves may lead to increased breaking time/energy expenditure, and may also result in chela damage for the crab. However, laboratory experiments have shown (Ropes 1968) that green crabs are able to open and consume soft-shell clams that were longer than the carapace width of the crabs. In this study, the mean length of the large soft-shell clams was 54.7 ± 6.79 (SD) mm, and only 17 of the 463 clams sampled were larger than the 65 mm carapace width of the crabs used. Therefore, shell thickness was probably not a factor in green crabs selecting small clams. We feel that burial depth of the clams was the basis for the selection of small clams by green crabs. Zaklan and Ydenberg (1997) found similar results in predation experiments on soft-shell clams by red rock crabs (*Cancer productus* Randall) and attributed large clam survival to a depth refuge. Larger soft-shell clams are usually found deeper in the sediment than small clams (Blundon & Kennedy 1982, Zaklan & Ydenberg 1997), and we commonly found large clams at depths of 30 cm when sampling. Even though green crabs can burrow effectively, digging pits up to 15 cm deep (Ropes 1968, Ropes 1988, Lindsay & Savage 1978); our results suggest that green crabs are not effective predators at the depths where large soft-shell clams reside.

Large Soft-shell Clam Population Structure

The large soft-shell clam population at our experimental site was dominated by the 4-year and 5-year age classes, with weak

year classes for clams aged 1, 2, and 3 years. Yearly variability in age class strength, attributed to changes in reproductive success, has been well documented in soft-shell clams (Kube 1996, MacKenzie & McLaughlin 2000, Strasser et al. 2001), but the weak year classes over the previous 3 years correspond with the period in which green crabs became abundant in Pomquet Harbour (P. J. Williams, unpublished data). An alternative explanation involves the recent outbreak of Hemic neoplasia that has been reported by McGladdery et al. (2001) in soft-shell clams in Atlantic Canada, however incidence of this disease was highest in northwestern Prince Edward Island and relatively low in the areas closest to Pomquet Harbour. We believe that green crab predation in the years leading up to our study may have strongly influenced the population structure of soft-shell clams in Pomquet Harbour. Both Glude (1955) and Welch (1968) report that green crabs effectively removed young-of-the-year soft-shell clams from affected beds. Reproductive output peaks late in life for soft-shell clams (Brousseau 1978), with larger clams providing most of the reproductive effort. In Pomquet Harbour, when the older cohorts senesce or die, larval supply to the bed will drastically decline.

Cage Artifacts

In any study involving the placement of structures on a mudflat, there is the potential for a variety of confounding physical and biologic effects (Hulberg & Oliver 1980). In this study, there was no significant difference in organic content between treatments, no noticeable scouring or deposition of sediment within the cages, and no post-experiment differences in sediment particle size distribution. The cages experienced little fouling and drift algal accumulation was minimal. These results suggest that cage-induced physical artifacts were minimized in this experiment.

Refuge and feeding by small epibenthic predators within cages is another possible confounding factor, and these types of organisms could have caused some of the mortality we observed in small soft-shell clams. We did not observe any such predators during biweekly checks or during the final sampling of the cages, however organisms such as small mummichogs (*Fundulus heteroclitus* Say), sand shrimp (*Crangon septemspinosa* Say), and small green and rock crab (*Cancer irroratus* Say) could have entered the cages at high tide, and exited prior to low tide. There are, however, several reasons why we feel that any impact of such predators on the experimental results was negligible. Mummichogs are very stout fish, and the 1-cm mesh-size of the cages would preclude fish larger than approximately 50 mm in length. Smaller fish could have preyed upon newly settled soft-shell clams in cages, but this

length is at the smaller range of fish reported to be effective predators on juvenile soft-shell clams (MacKenzie & McLaughlin 2000). We observed very few sand shrimp or rock crab at the site at any time. Finally, if predators such as these had removed small soft-shell clams, one would expect that they would remove similar numbers from all cages. If anything, the only bias one might expect would be for predators to avoid cages with green crabs, thereby making it more difficult to demonstrate a significant effect in green crab enclosure cages.

Clearly the confinement of the crabs in the D1 and D2 cages contributed to the crab mortality that occurred. The bulk (19 of 21) of the mortalities took place in the 20 days prior to the end of the experiment, from August 3 to August 23, 2001. Two crabs died in the D1 cages, in the absence of other crabs, suggesting the combination of starvation and the high daytime temperatures during this period could have contributed to the mortality. Cannibalism was likely a factor in the higher mortality observed in the D2 cages.

Aquaculture Implications

Our results suggest that soft-shell clam aquaculture operations in the southern Gulf of St. Lawrence should protect small soft-shell clams until they reach an appropriate size and depth refuge whereby they cannot be attacked by green crabs. If green crabs are allowed to prey freely on soft-shell clam beds, they will probably decimate small clam stocks and eventually cause steep population declines. Because adult soft-shell clams seem to have a depth refuge from predation, the effects of a decline of small clams may not be noticed until well after the onset of the green crab invasion, when the older, stronger year classes start dying off and are not replaced. Recreational or commercial harvesters only retain large clams, and may not immediately notice a reduction in numbers of small clams. Excluding green crabs by fencing is an effective mechanism for preservation of small soft-shell clams (Glude 1955, Beal & Kraus 2002). With the current expansion of green crabs into areas of heavy bivalve aquaculture, such a precaution may be needed to preserve harvested populations.

ACKNOWLEDGMENTS

The authors thank Ashley Bouchie, Sarah Fraser, Sean Mitchell, and Jack MacNeil for assistance with the field work and Peter Kaiser, Dr. Barry Taylor, Dr. Mike Melchin, and Dr. David Garbary for technical assistance. We also thank NSERC for the operating grant used to fund this research.

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IN SITU AQUACULTURE PROTOCOL FOR TWO TROPICAL PECTINIDS, *BRACTECHLAMYS VEXILLUM* (REEVE 1853) AND *MIMACHLAMYS GLORIOSA* (REEVE 1853): SPAT COLLECTION, REARING, GROWTH AND MORTALITY

LAURENT WANTIEZ¹* AND PIERRE THOLLOT²

¹L'ERREM, University of New Caledonia, BP 4477, 98847 Noumea cedex, New Caledonia; ²deceased

ABSTRACT The aim of this study is to propose an *in situ* aquaculture protocol, including spat collecting and rearing, for two tropical species of Pectinids (*Bractechlamys vexillum* and *Mimachlamys gloriosa*). Spat were collected using small mesh bags (80 × 40 cm) filled with used fishing nets and fixed on lines 1.5 and 2.5 m above the sea bottom. The collecting lines were set above natural stocks during 2 months in 2 locations, and surveyed every 2 weeks. On average, 118 *Bractechlamys vexillum* (mean size 8.08 mm) and 132 *Mimachlamys gloriosa* (mean size 10.17 mm) were collected in each bag. The height of the bags above the sea bottom did not affect the collecting yield or the mean size of juveniles with either species. Predation was not directly evaluated in the bags but regular visual observations showed that it remained anecdotic during the 2-month collecting period. After 2 months, the juveniles were transferred to Japanese lantern nets (1.5 × 0.4 m) for rearing, each lantern containing ten levels. Lanterns were deployed in 2 locations and surveyed every 2 weeks for a period of 18 months. Two initial densities (400 individuals/m² and 800 individuals/m²) were studied for each species. Growth (Von bertalanffy model) of the two species was not affected by the tray position in the lantern. Density did not affect significantly K parameter but specimens reached a larger size in the low-density lantern. Growth of *Bractechlamys vexillum* was related to the site (K varied between 0.073 and 0.088 per week; H_∞ varied between 45.5 and 42.3 mm), with a lower K value but a bigger size in Baie des Citrons². Growth of *Mimachlamys gloriosa* was not linked to the site (K = 0.066 per week, H_∞ = 57 mm). *Bractechlamys vexillum* and *Mimachlamys gloriosa* were characterized by significant variations in mortality rates (20% to 70%) after 18 months of rearing. The influence of density was less conclusive. *Bractechlamys vexillum* displayed a higher mortality in the low-density lanterns and *Mimachlamys gloriosa* a lower mortality in low-density lanterns. The site had a similar effect on mortality rates for both species, with higher mortality in Baie des Citrons². Spat transfer losses only affected *Bractechlamys vexillum*. Diseases and biologic traits also affected the populations and mortality rates were variable between levels. Mortality models gave lower Z estimates for reared populations (0.16–0.43 per year for *Bractechlamys vexillum* and 0.16–0.55 per year for *Mimachlamys gloriosa*) than for natural populations. In conclusion, *in situ* aquaculture of the studied species is feasible and optimized protocols are proposed for spat collecting and rearing. Commercial size is reached after 12 months of rearing (43.2 mm for *Bractechlamys vexillum* and 54.8 mm for *Mimachlamys gloriosa*) with survivorship of 66% to 85% for *Bractechlamys vexillum*, and 58% to 86% for *Mimachlamys gloriosa* depending on density and site.

KEY WORDS: aquaculture, Pectinids, spat collecting, rearing, growth, mortality, New Caledonia, *Bractechlamys*, *Mimachlamys*

INTRODUCTION

Tropical pectinids are common in the New Caledonian lagoon (Lefort 1991). Six species have a commercial value and constitute exploitable stocks (Lefort 1991). However, the use of bottom trawled fishing devices (dredges and trawls) is forbidden by the Southern Province Authorities in all of the lagoon area because of their destructive impact on sea bottom habitats (Hutchings 1990). Because there is a demand for these shellfish on the local market, a temporary authorization was given to a commercial fisherman to collect pectinids using scuba diving gear between 1993 and 1995. This authorization was not renewed and today these stocks can only be exploited by snorkeling. Because this technique is laborious, has limited efficiency, and gives low collecting yields, it is only anecdotal and the resource is not exploited. A possible way of exploiting these organisms, which uses neither destructive fishing gear nor scuba diving, is to develop a simple *in situ* aquaculture protocol.

The aim of this study is to propose an *in situ* aquaculture protocol for two tropical pectinids, *Bractechlamys vexillum* (Reeve 1853) and *Mimachlamys gloriosa* (Reeve 1853). This protocol includes spat collection and juvenile rearing up to commercial size. Site selection, technical design, and methodology are described in

the present article. Spat availability and juvenile growth and mortality have been studied in relation to site characteristics and rearing density.

MATERIALS AND METHODS

Species studied

Two pectinid species were selected for this study, *Bractechlamys vexillum* (Reeve 1853) and *Mimachlamys gloriosa* (Reeve 1853). These species, which are of commercial interest, are the most common species in the South West lagoon of New Caledonia (Lefort 1991). *Bractechlamys vexillum* is a mobile species, which lives exclusively on soft-bottoms and concentrates in hollows, under *Halimeda* algae or close to small coral heads or sponges (Chauvet 2000). *Mimachlamys gloriosa* lives fixed on algae (*Avrainvillea nigricans*, *Halimeda cylindracea*), antipatharia (*Cirrhipathes*) or on hard substrate. Spawning of these species occurs all year long with more intensity in summer than in winter (Lefort & Clavier 1992), but the spat collecting yield is higher in winter (Chauvet 2000). Douchement and Chauvet (1994) and Chauvet (2000) showed that it was easily possible to collect wild spat for these two species.

Spat collecting

To successfully collect spat, the sites selected must be located near natural stocks with mature individuals (Douchement & Chau-

The Southern Province of New Caledonia Department of Living Resources funded this study.

vet 1994). Two sites located in bays near Nouméa (New Caledonia) were selected for spat collection, Nouville1 and Baie des Citrons1. Prior to the deployment of the collectors, the local pectinid populations were sampled in both sites. Nouville1, depth 11–13 m, has a muddy-sand bottom with some rocks, and is colonized by a typical "lagoon grey bottom" community (as defined by Richer de Forges et al. 1987) with algae (i.e. *Caulerpa*, *Halimeda*, *Sargassum*, *Udotea*), sponge, free-living corals (i.e. *Cyloseris cyclolites*, *Heteropsammia machelini*, *Trachiphyllia geofroyi*), gastropods (i.e. *Strombus luhuanus*) and holothurians (i.e. *Holothuria edulis*, *H. scabra*, *Stichopus variegatus*). *Mimachlamys gloriosa* was the more abundant of the two pectinids (70%) on this site, with individuals fixed upon *Halimeda*, sponges or hard substrate (shells fragments and dead corals). Baie des Citrons1, depth 11 m, has a uniform muddy-sand substrate, colonized by a "lagoon grey bottom" community quite similar to Nouville1. However, *Bractechlamys vexillum* was, by far, the more abundant of the two pectinids on this site (more than 90%), with individuals being partly buried in the sand or laying on the bottom.

Spat collecting devices were bags (80 × 40 cm) made of 50% agricultural shadowing curtain and filled with 6–8 m² of used nylon fishing nets of 3–6 cm square mesh size. The agricultural curtain has a 6 mm square mesh size, which allows the larvae to penetrate into the bag and protect the juveniles from external predators. Each collecting structure was made of two devices fixed onto a vertical rope, 1.5 m and 2.5 m above the bottom (Fig. 1). Ten collecting structures were set at each site, with nine of them surrounding a central one, each structure being at least 20 m apart. This design was adopted to optimize spat collection without knowledge of local current patterns. The collecting structures were set in August, which is the most favorable season according to Douchement and Chauvet (1994). They were controlled and fouling was removed from external bags twice a month. After 2 months, the juveniles were detached from the collectors and transferred to the rearing structures, as recommended by Douchement and Chauvet (1994). This collecting schedule is satisfactory to collect juveniles large enough for the rearing structure but not long enough for predators to develop inside the collecting bags and

consume the juveniles. Each juvenile transferred to the rearing structure was measured (shell height) to the nearest millimeter.

Rearing

The environmental characteristics of the rearing sites must be compatible with the growth of pectinids. Two rearing sites were selected, Nouville2 and Baie des Citrons2. Nouville2 is a lagoonal site, depth 20 m, and has a fine-sand bottom with a thin muddy layer on top. No algae lived on the bottom and epigeal macrobenthos was scarce, with only a few *Holothuria leucospilota*. Baie des Citrons2 is located in a coastal bay, depth 11 m, and has a muddy-sand bottom and was colonized by a "lagoon grey bottom" community quite similar to Baie des Citrons1.

Juveniles were grown in Japanese lantern nets (height 1.5 m). Each lantern was made of 10 trays (40 cm diameter) linked by three ropes resistant to heavy weight and protected by a net of 5 cm square mesh size. The trays were 15 cm apart. The protecting net allowed water and nutritive particles to penetrate to ensure the growth of pectinids, and prevent external predators from reaching the juveniles. This is a standard rearing technique widely used to grow similar species throughout the world (Imai 1980, Buestel et al. 1982, Dupony 1983, Lucien-Brun & Lachaux 1983, Lovatelli 1987, Barnabé 1989, Dao 1989, Doumenge 1989, among others). Four lanterns were fixed to a rearing structure at each site (Fig. 2).

At each site, one low and one high-density lantern were set for each species to test two initial rearing densities: 400 individuals/m² (50 individuals per tray) and 800 individuals/m² (100 individuals per tray). The lanterns were checked every 2 weeks, fouling was removed if necessary, as all potential predators (mainly crabs), which recruited in the lantern, and dead pectinids. All living specimens were counted and shell height was measured every month for 18 months. Handling operations were restricted as much as possible to avoid physiologic stress, individuals being always kept in water when removed from the trays.

Data analysis

A three way Anova and *post hoc* tests (Sokal & Rohlf 1981) were used to test the impact of species, site and height above sea bottom on spat collecting yields.

A Von bertalanffy growth model (Sparre et al. 1989) was calculated for *Bractechlamys vexillum* and *Mimachlamys gloriosa*:

$$H_t = H_{\infty}(1 - e^{-K(t-t_0)})$$

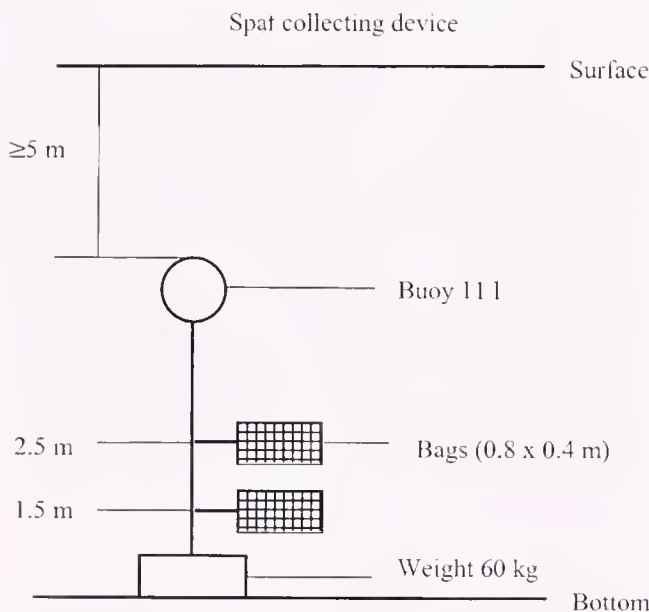


Figure 1. Design of spat collecting devices.

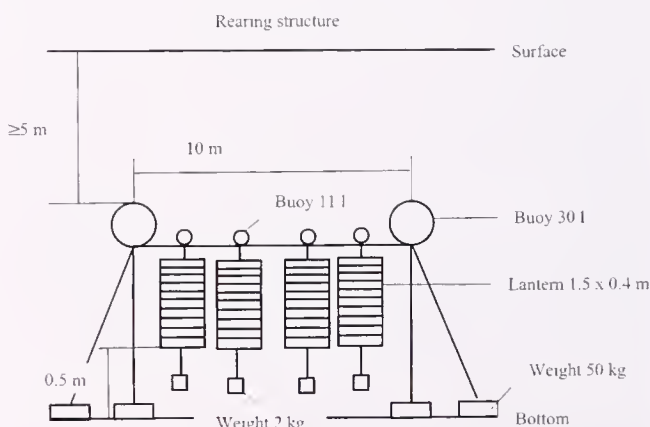


Figure 2. Design of rearing structures.

where H_t shell height at age t (mm); H_∞ maximum shell height (mm); K curvature parameter (per week); t age (week); t_0 initial condition parameter. These parameters were estimated by the Gulland and Holt method (Sparre et al. 1989), using shell size increases between two successive samplings. Both growth parameters were derived from a linear regression:

$$\frac{\Delta H}{\Delta t} = KH_\infty - KH_t$$

where ΔH shell height increase between two samplings (mm); Δt time between two samplings (week). The initial condition parameter (t_0) was set to zero. K parameters were compared using the test for equality of slopes and unplanned comparisons among a set of regression coefficients (Sokal & Rohlf 1981).

Instantaneous and cumulated mortality rates (MI) were calculated between two sampling dates:

$$MI_{i+1} = \frac{N_i - N_{i+1}}{N_i}$$

where MI_{i+1} instantaneous mortality rate at $t = i+1$; N_i number of living specimens at $t = i$; N_{i+1} number of living specimens at $t = i+1$. Cumulated mortality rates were calculated from the beginning of the rearing phase:

$$MC_i = \frac{N_0 - N_i}{N_0}$$

where MC_i cumulated mortality at $t = i$; N_0 Initial number of specimens. A Mortality model was calculated:

$$N_t = N_0 e^{-Zt}$$

where N_t Number of living specimens at age t ; Z instantaneous rate of total mortality (per year). These parameters were estimated using a linear regression after logarithm transformation and compared using the test for equality of slopes and unplanned comparisons among a set of regression coefficients (Sokal & Rohlf 1981).

RESULTS

Spat collecting

The mean number of spat larger than 7 mm was 118 ± 38 specimens per bag for *Bractechlamys vexillum* and 132 ± 38 specimens per bag for *Mimachlamys gloriosa*, for both sites. A significant interaction occurred between site and species (Anova, $P < 0.01$), which did not allow us to test these two factors separately. This interaction was a result of significantly higher yields per collector in Nouville1 for *Bractechlamys vexillum* (Mann-Whitney test, $P < 0.05$, Table 1) in spite of the dominance of *Mimachlamys gloriosa* adult populations at this site. The opposite pattern was observed in Baie des Citrons1 with significantly higher yields per collector for *Mimachlamys gloriosa* (Mann-Whitney test, $P < 0.05$, Table 1) in spite of the dominance of *Bractechlamys vexillum* adult populations. The height of the collecting bag above the sea bottom had no significant effect on collecting yields (Anova, $P > 0.05$, Table 1). However, the general trend was higher collecting yields in the lower bag (Table 1). Regular visual observation during fouling removal and the absence of predators big enough to eat the spat inside the bags at the end of the collecting phase, showed that spat loss was negligible during the 2-month period of spat collecting.

The mean shell height of collected juveniles was 8.08 mm for

TABLE 1.

Collecting yield per species, site and collector for specimens of more than 7 mm.

Site/Position	<i>Bractechlamys vexillum</i>	<i>Mimachlamys gloriosa</i>
Nouville1		
High	157 (113–214)	120 (53–188)
Low	233 (20–343)	87 (38–160)
Mean	190 \pm 110	103 \pm 26
Baie des Citrons1		
High	55 (17–91)	140 (20–343)
Low	106 (15–150)	225 (20–343)
Mean	87 \pm 26	183 \pm 97
Mean	118 \pm 38	132 \pm 38

Mean number of specimens per bag (minimum and maximum between brackets). 95% confidence intervals are given. High: collectors fixed 2.5 m above bottom. Low: collectors fixed 1.5 m above bottom.

Bractechlamys vexillum (max 14 mm) and 10.17 mm for *Mimachlamys gloriosa* (max 17 mm). A significant interaction occurred between site and species (Anova, $P < 0.01$), which did not allow us to test these two factors separately. This interaction was a result of *Mimachlamys gloriosa* having a significantly larger size on both sites (t -Student test, $P < 0.001$, Table 2) and specimens having a larger shell height in Nouville1 than in Baie des Citrons1 for each species (t -Student test, $P < 0.01$, Table 2). The height of the collecting bag above the sea bottom had no significant effect on juvenile size (Anova, $P > 0.05$, Table 2).

The size frequency distributions of juveniles bigger than 7 mm, are similar for both species (Fig. 3). Distributions were unimodal with a left skewness due to minimum size selection (Fig. 3). Size variation among specimens was low for both species (CV lower than 12%). The two most important size classes included 57–79% of the specimens, with the exception of *Mimachlamys gloriosa* in Nouville2 where these two classes only included 42% of the specimens (Fig. 3). However, the size frequency distribution differed slightly between sites (Fig. 3), with a 1-mm lag in Baie des Citrons2 compared with Nouville2.

Rearing and growth of *Bractechlamys vexillum*

Bractechlamys vexillum size increased regularly on all trays to reach a mean size per tray between 38 and 53 mm after 18 months.

TABLE 2.

Mean shell height of collected specimens of more than 7 mm per species, site and collector.

Site/Position	<i>Bractechlamys vexillum</i>	<i>Mimachlamys gloriosa</i>
Nouville1		
High	8.55 (13)	10.79 (17)
Low	8.30 (14)	10.75 (16)
Mean	8.44 (14)	10.77 (17)
Baie des Citrons1		
High	8.06 (11)	9.17 (12)
Low	7.85 (11)	9.08 (14)
Mean	7.93 (11)	9.12 (14)
Mean	8.08 (14)	10.17 (17)

Mean shell height per bag (maximum between brackets). High: collectors fixed 2.5 m above bottom. Low: collectors fixed 1.5 m above bottom.

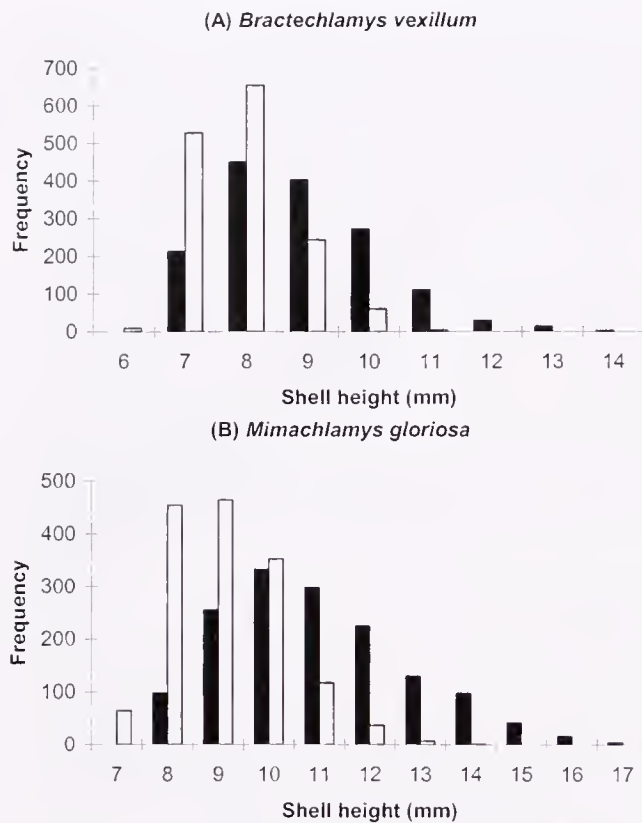


Figure 3. Size frequency distribution of the collected juveniles per species (A and B) and per site (black: Nouville1; white: Baie des Citrons1).

Daily increase rates were higher at the beginning of the rearing period (maximum 0.34 mm/day) and decreased rapidly to become very low after 200–250 rearing days (< 0.05 mm/day). A growth model was calculated for each tray to test the effect of tray position on growth. The theoretical maximum shell height (H_{∞}) cannot be statistically compared, and no clear pattern was observed with tray position (Table 3). Curvature parameters, which determine how fast the specimens approach maximum size, were compared among trays of each lantern (Table 3). Differences were not significant for low-density lanterns (comparison of regression coefficients, $P > 0.05$). Significant differences occurred in high-density lanterns (unplanned comparison of regression coefficients, $P < 0.05$, Table 3). However, the link between *Bractechlamys vexillum* growth and tray position was not interpretable. Consequently, all trays were pooled to test the effect of density on growth.

Similar patterns were observed for shell height and daily growth rates (Fig. 4). However, *Bractechlamys vexillum* reached a larger size in low-density lanterns (47.4 mm in Baie des Citrons2 and 43.6 mm in Nouville2) than in high-density ones (42.9 mm in Baie des Citrons2 and 40.6 mm in Nouville) (Student test, $P < 0.001$). A growth model was calculated for each lantern to test the effect of density on growth (Table 3). H_{∞} was higher for low-density lanterns (47.9 mm in Baie des Citrons2 and 44.0 mm in Nouville2) than for high-density ones (43.2 mm in Baie des Citrons2 and 40.6 mm in Nouville2). However, there was no significant difference between curvature parameters of low and high-density lanterns (comparison of regression coefficients, $P > 0.05$). Consequently, low and high-density lanterns were pooled to test the effect of site on growth.

TABLE 3.

Von Bertalanffy growth model parameters per tray, density and site for *Bractechlamys vexillum*.

Low Density Tray	Nouvelle2		High Density Tray	Nouvelle2	
	K	H_{∞}		K	H_{∞}
1	0.064	43.5	2	0.068	41.2
5	0.071	42.6	9	0.076	42.3
2	0.071	42.6	1	0.076	40.4
6	0.072	44.8	3	0.079	40.2
3	0.072	41.3	4	0.079	40.2
8	0.074	44.4	8	0.079	41.3
7	0.075	44.9	5	0.082	40.1
9	0.076	46.3	10	0.082	42.3
4	0.076	39.5	6	0.085	39.5
10	0.079	45.2	7	0.086	39.3
All trays	0.082	44.0	All trays	0.095	40.6

Low Density Tray	Baie des Citrons2		High Density Tray	Baie des Citrons2	
	K	H_{∞}		K	H_{∞}
1	0.054	49.1	1	0.054	45.7
5	0.055	50.0	8	0.055	45.0
4	0.058	47.8	9	0.060	43.7
6	0.058	48.8	7	0.066	44.2
10	0.059	54.6	2	0.067	42.7
8	0.061	47.8	4	0.068	41.8
2	0.061	45.9	6	0.069	43.1
7	0.066	46.7	3	0.069	42.2
9	0.067	46.8	5	0.070	41.2
3	0.067	46.4	10	0.074	43.7
All trays	0.070	47.9	All trays	0.075	43.2

Trays are ranked per K values. K, curvature parameter (per week); H_{∞} , Maximum shell height (mm).

Vertical lines regroup K values not significantly different (unplanned comparison of regression coefficients, $P > 0.05$).

Similar patterns were observed for shell height and daily increase rates at both sites, despite a larger size in Baie des Citrons2 (44.5 mm) than in Nouville2 (41.3 mm) (Student test, $P < 0.001$). A growth model was calculated for each site (Fig. 4). Baie des Citrons2 was characterized by a significantly lower growth rate (comparison of regression coefficients, $P < 0.05$) and a larger maximum size ($K = 0.073$ per week, $H_{\infty} = 45.5$ mm) than Nouville2 ($K = 0.088$ per week, $H_{\infty} = 42.3$ mm) (see Fig. 4). Growth was similar on both sites during the initial growth phase (up to 35 mm) and differed after 6 months of rearing (Fig. 4).

Rearing and growth of *Mimachlamys gloriosa*

Mimachlamys gloriosa size increased regularly on all trays to reach a mean size per tray of between 51 and 60 mm after 18 months. Daily increase rates decreased rapidly and became very low after 250 to 300 rearing days (< 0.05 mm/day). A growth model was calculated for each tray to test the effect of tray position on growth. The theoretical maximum shell height cannot be statistically compared, and no clear pattern was observed with tray position (Table 4). Curvature parameters were compared among trays of each lantern (Table 4). The differences were not significant (comparison of regression coefficients, $P > 0.05$). Tray posi-

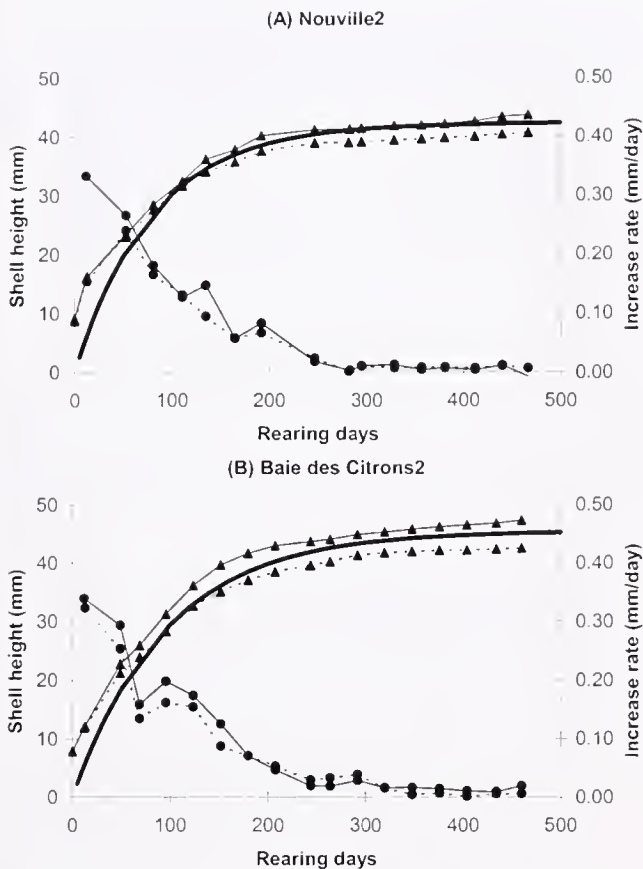


Figure 4. Shell height (▲), daily increase rates (●) and von Bertalanffy growth model (thick line) of *Bractechlamys vexillum* in Nouville2 (A) and Baie des Citrons2 (B). Continuous lines represent high-density lantern and broken lines represent low-density lantern.

tion had no significant effect on growth. Consequently, all trays were pooled to test the effect of density on growth.

Similar patterns were observed for shell height and daily growth rates (Fig. 5). However *Mimachlamys gloriosa* reached a larger size in low-density lanterns (59.1 mm in Baie des Citrons2 and 55.2 mm in Nouville2) than in high-density ones (53.9 mm in Baie des Citrons2 and 54.4 mm in Nouville2) (Student test, $P < 0.001$ in Baie des Citrons2 and $P < 0.05$ in Nouville2). A growth model was calculated for each lantern to test the effect of density on growth (see Table 4). H_{∞} was higher for low-density lanterns (60.7 mm in Baie des Citrons2 and 56.7 mm in Nouville2) than for high-density ones (54.1 mm in Baie des Citrons2 and 54.5 mm in Nouville2). However, there was no significant difference between curvature parameters of low and high-density lanterns (comparison of regression coefficients, $P > 0.05$). Consequently, low and high-density lanterns were pooled to test the effect of site on growth.

Similar patterns were observed for shell height and daily growth rates, and *Mimachlamys gloriosa* reached the same mean size (57 mm) on both sites. A growth model was calculated for each site (Fig. 5). Growth parameters calculated on the 2 sites were very close ($K = 0.069$ per week $H_{\infty} = 56.2$ mm in Nouville2 and $K = 0.063$ per week $H_{\infty} = 57.4$ mm in Baie des Citrons2). Curvature parameters were not significantly different (comparison of regression coefficients, $P > 0.05$). Growth was similar on both sites during all the rearing period (see Fig. 5). Consequently, a

TABLE 4.

Von Bertalanffy growth model parameters per tray, density and site for *Mimachlamys gloriosa*.

Low Density Tray	Nouville2		High Density Tray	Nouville2	
	K	H_{∞}		K	H_{∞}
1	0.056	56.2	6	0.055	54.5
8	0.062	57.2	3	0.057	53.6
2	0.063	54.9	2	0.057	51.8
4	0.063	56.6	1	0.058	55.5
7	0.064	58.1	7	0.058	55.1
6	0.064	56.3	10	0.061	56.5
3	0.065	56.1	8	0.062	55.7
5	0.065	57.7	4	0.062	53.6
10	0.068	56.3	6	0.055	54.5
9	0.068	56.6	5	0.063	53.9
All trays	0.062	56.7	All trays	0.064	54.5

Low Density Tray	Baie des Citrons2		High Density Tray	Baie des Citrons2	
	K	H_{∞}		K	H_{∞}
5	0.048	61.9	1	0.058	55.5
9	0.051	62.1	8	0.054	54.8
10	0.051	61.2	2	0.051	54.7
1	0.052	58.1	4	0.056	53.2
4	0.053	60.2	7	0.057	53.7
3	0.053	59.9	6	0.053	54.3
7	0.053	59.9	3	0.053	55.5
2	0.054	60.9	5	0.055	52.9
8	0.055	61.3	10	0.058	51.7
6	0.058	59.6	9	0.056	56.5
All trays	0.074	60.7	All trays	0.068	54.1

Trays are ranked per K values. K, curvature parameter (per week); H_{∞} , Maximum shell height (mm).

K values were not significantly different (comparison of regression coefficients, $P > 0.05$).

global Von bertalanffy growth model was calculated: $K = 0.066$ per week and $H_{\infty} = 57$ mm.

Mortality of *Bractechlamys vexillum*

Instantaneous mortality rates (MI) of *Bractechlamys vexillum* per tray were so heterogeneous and variable that the mortality model was only applicable for 29 of the 40 trays (Table 5). Instantaneous rates of total mortality (Z) calculated for these trays were significantly different (unplanned comparison of regression coefficients, $P < 0.05$) (Table 5). However, the effect of tray position was not interpretable (Table 5). Consequently, data from each tray of a lantern were pooled to test the effect of density and site on mortality.

Two periods were characterized by high mortality rates (Fig. 6). The first one took place just after the transfer of juveniles from collecting devices, particularly on low-density lanterns (Fig. 6). After this first period, the instantaneous mortality rates decreased greatly and varied between 0 and 10%. These rates increased at the end of the rearing period, particularly in the Nouville2 low-density lantern and the Baie des Citrons2 high-density lantern (Fig. 6). Punctual high mortality rates were also observed on a few trays, between 150 and 250 rearing days. Cumulated mortality rates increased regularly (Fig. 6). In Nouville2, the final cumulated mor-

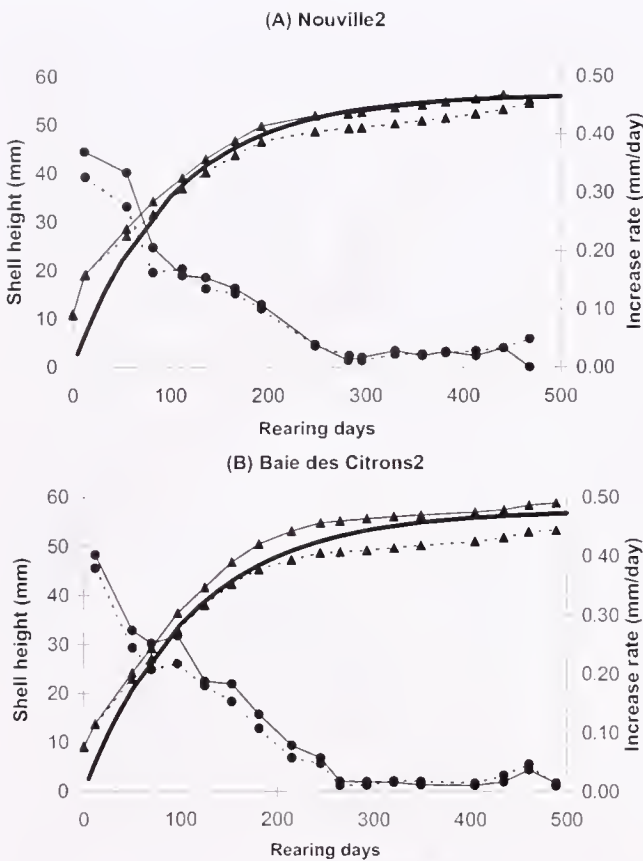


Figure 5. Shell height (▲), daily increase rates (●) and von Bertalanffy growth model (thick line) of *Mimachlamys gloriosa* in Nouville2 (A) and Baie des Citrons2 (B). Continuous lines represent high-density lantern and broken lines represent low-density lantern.

tality rate was higher in the low-density lantern (58%) than in the high-density one (21%) (Fig. 6). Density had a significant effect on the modeled mortality (Fig. 7). The instantaneous rate of total mortality (Z) was significantly higher in low-density lanterns for each site (comparison of regression coefficients, $P < 0.05$) (Table 5; Fig. 7). Consequently, the effect of site was tested separately for each density level.

For the low-density lanterns, instantaneous rates of total mortality (Z) were not significantly different between sites (comparison of regression coefficients, $P > 0.05$, Table 5, Fig. 7). Conversely, differences were significant for high-density lanterns with lower rates in Nouville2 (comparison of regression coefficients, $P < 0.05$, Table 5, Fig. 7). Despite significant effects of rearing density and site on mortality of *Bracteacclamys vexillum*, mortality models were quite similar for the 2 sites and density levels, with the exception of the high-density lantern in Nouville2 characterized by low mortality rates (Fig. 7).

Mortality of *Mimachlamys gloriosa*

Instantaneous mortality rates (M) of *Mimachlamys gloriosa* satisfied the mortality model for all trays. Significant differences in instantaneous rates of total mortality (Z) occurred between trays (comparison of regression coefficients, $P < 0.05$, Table 6). Z was significantly higher in tray n°1 of the high-density lantern in Baie des Citrons2, and high values characterized n°1 tray of the other lanterns (unplanned comparison of regression coefficients, $P <$

TABLE 5.

Instantaneous rates of total mortality (Z, per year) per tray, density and site for *Bracteacclamys vexillum*.

Nouville2				Baie des Citrons2			
Low Density		High Density		Low Density		High Density	
Tray	Z	Tray	Z	Tray	Z	Tray	Z
8	0.13	8	0.07	9	0.23	7	0.17
6	0.21	5	0.07	3	0.31	6	0.25
5	0.55	3	0.07	2	0.32	9	0.27
2	0.57	6	0.12	4	0.39	3	0.28
4	0.84	2	0.16	6	0.40	2	0.28
3	0.94	7	0.20	5	0.49	8	0.29
1	—	9	0.25	1	0.58	4	0.38
7	—	1	—	7	—	5	0.47
9	—	4	—	8	—	1	1.06
10	—	10	—	10	—	10	—
All trays	0.40		0.16	All trays	0.43		0.37

Trays are ranked per Z values. —: mortality model not respected. Vertical lines regroup Z values not significantly different (unplanned comparison of regression coefficients, $P > 0.05$).

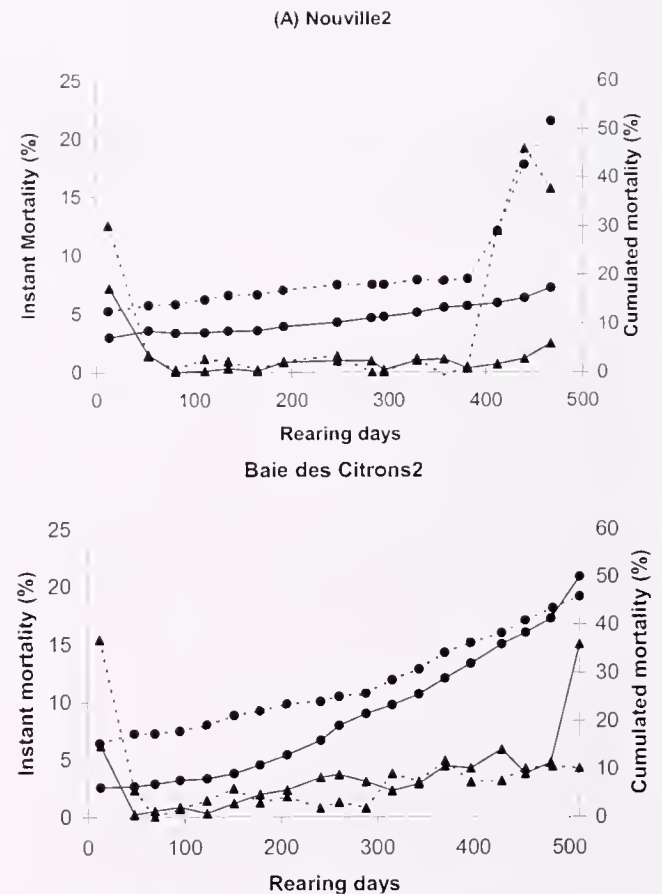


Figure 6. Instant (▲) and cumulated (●) mortality rates of *Bracteacclamys vexillum* in Nouville2 (A) and Baie des Citrons2 (B). Continuous lines represent high-density lantern and broken lines represent low-density lantern.

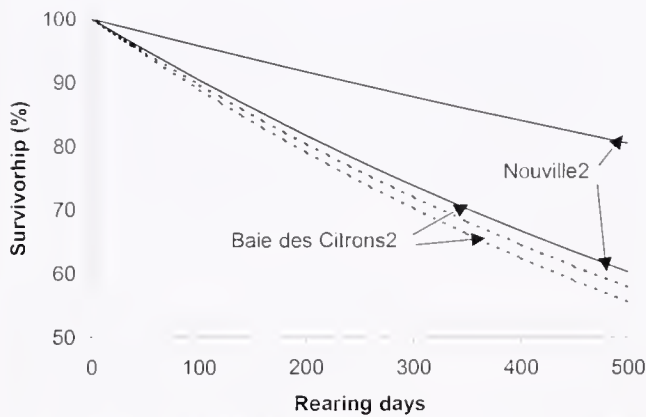


Figure 7. Modeled survivorship of *Bractechlamys vexillum* by density and site. Model parameters are given in Table 5. Continuous lines represent high-density lantern and broken lines represent low-density lantern.

0.05, Table 6). The variations in mortality on the other trays were not linked to tray position (Table 6). Data from all trays of each lantern were pooled to test the effect of density on mortality.

No significant mortality was observed after the transfer of juveniles and instantaneous mortality rates (MI) were low at the beginning of the rearing period (Fig. 8). These rates increased after 250 to 300 rearing days and were higher at the end of the rearing period. At the end of the rearing period, cumulated mortality rates were close to 50% for low-density lanterns and 70% for high-density lanterns in Baie des Citrons2, and reached 29% for low-density lanterns and 39% for high-density lanterns in Nouville2 (see Fig. 8). Density had a significant effect on modeled mortality. Instantaneous rates of total mortality (Z) were higher on high-density lanterns (comparison of regression coefficients, $P < 0.05$, Table 6, Fig. 9). Consequently, the effect of site was tested separately for each density level.

Instantaneous rates of total mortality (Z) were significantly higher in Baie des Citrons2 than in Nouville2 for each density level (comparison of regression coefficients, $P < 0.05$, Table 6)

TABLE 6.

Instantaneous rates of total mortality (Z, per year) per tray, density and site for *Mimachlamys gloriosa*.

Nouville2				Baie des Citrons2			
Low Density		High Density		Low Density		High Density	
Tray	Z	Tray	Z	Tray	Z	Tray	Z
5	0.08	4	0.20	5	0.19	4	0.46
3	0.11	3	0.20	4	0.24	5	0.47
8	0.11	7	0.21	7	0.26	10	0.47
6	0.13	2	0.22	2	0.27	6	0.47
4	0.13	5	0.23	3	0.28	7	0.49
10	0.15	6	0.23	9	0.30	8	0.53
9	0.18	8	0.27	8	0.30	3	0.54
2	0.21	9	0.32	10	0.34	2	0.55
7	0.21	1	0.33	6	0.37	9	0.72
1	0.28	10	0.39	1	0.38	1	0.94
All trays	0.16		0.26	All trays	0.29		0.55

Trays are ranked per Z values. Vertical lines regroup Z values not significantly different (unplanned comparison of regression coefficients, $P > 0.05$).

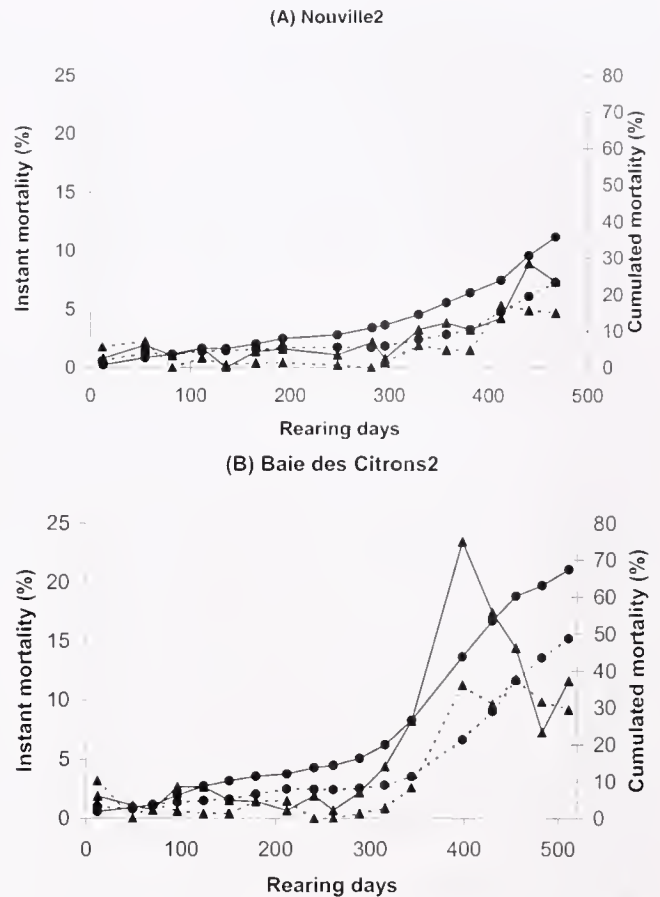


Figure 8. Instant (\blacktriangle) and cumulated (\bullet) mortality rates of *Mimachlamys gloriosa* in Nouville2 (A) and Baie des Citrons2 (B). Continuous lines represent high-density lantern and broken lines represent low-density lantern.

indicating a better survival in Nouville2 than in Baie des Citrons2 (Fig. 9).

DISCUSSION

Spat collecting

During this study nearly all the juveniles that recruited in the collecting bags were *Bractechlamys vexillum* and *Mimachlamys*

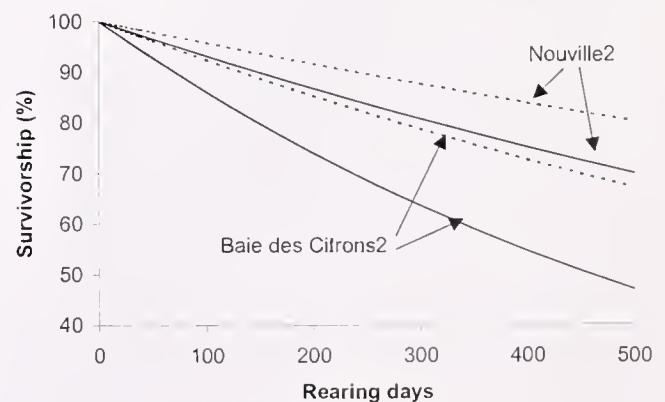


Figure 9. Modeled survivorship of *Mimachlamys gloriosa* by density and site. Model parameters are given in Table 6. Continuous lines represent high-density lantern and broken lines represent low-density lantern.

gloriosa, as observed by Chauvet (2000). Spat collecting yields were similar to those reported by Douchement and Chauvet (1994) in Baie des Citrons (114.7 juveniles of more than 7 mm per bag) for *Mimachlamys gloriosa*. Conversely, these authors reported lower yields for *Bractechlamys vexillum* (21.6 juvenile of more than 7 mm per bag in Baie des Citrons) than in this study. These differences probably reflect inter-annual variations in recruitment, as both studies were completed during the same season, which, according to Chauvet (2000) is the best recruitment season. This author also reported highly variable collecting yields per bag as in this study.

The results of this indicate an interaction between site and collected species. Such interaction was also observed by Douchement and Chauvet (1994). The presence of an adult population at a collecting site increase chances of spat recruitment but not necessarily high collecting yields. Other significant factors also affect collecting yields. Current patterns are important for invertebrate larval dispersal and recruitment even for species with a short plankton larval phase (Shanks et al. 2003), such as the common Pectinids in the New Caledonian lagoon (up to 6 days for *Bractechlamys vexillum* and 15 days for *Mimachlamys gloriosa*, Lefort & Clavier 1992). These patterns probably had a significant effect, on the higher collecting yields of *Bractechlamys vexillum* at Nouville1, despite the higher abundance of *Mimachlamys gloriosa*, and the opposite pattern observed at Baie des Citrons1. Douchement and Chauvet (1994) observed an opposite pattern than in this study, with collecting yields being nearly 3 times higher for *Mimachlamys gloriosa* than *Bractechlamys vexillum* in Baie des Citrons. Their results indicate that other factors may intervene.

Chauvet (2000) reported low collecting yields near the sea bottom, higher yields up to 2.5–3 m above the bottom and decreasing yields above 3 m. These results are similar to those for temperate scallop species (Brand et al. 1980, among others). During this study, collectors were installed in a narrow range, as recommended by Douchement and Chauvet (1994), that explained the absence of significant effect of this factor on collecting yields.

The approach of limiting the collecting phase to 2 months successfully prevented the impact of predators in the bags. This impact is known to be significant for temperate species when the collecting period is too long (Brand et al. 1980; Thouzeau 1991). During this study, crabs had recruited in the bags but they did not have time to grow large enough to deplete the juvenile populations of Pectinids.

The duration of the collecting phase was long enough to obtain juveniles at a size suitable to be transferred to the subsequent culture phase (size >7 mm) for the two species studied. Maximum juvenile sizes were similar to those of Douchement and Chauvet (1994) after similar collecting phases for both species. Mean sizes were also similar for *Bractechlamys vexillum* but the mean size of *Mimachlamys gloriosa* was smaller (<9 mm) in the study of Douchement and Chauvet (1994). Post recruitment growth was faster for *Mimachlamys gloriosa* (10.6 mm/month) than for *Bractechlamys vexillum* (8.6 mm/month) (Chauvet 2000), which explained the differences observed in the mean size of juveniles between the two species at the end of the collecting phase. The mean size of juveniles was also larger in Nouville1 than in Baie des Citrons1 during this study because of the sampling schedule. Bags located in Nouville1 were collected 5 days after those in Baie des Citrons1. During these 5 days juveniles continued to grow and the differences in mean sizes corresponded to the growth rates

given by Chauvet (2000). This is confirmed by the differences in size frequency distributions between sites for both species. An extended recruitment phase could explain the difference in size frequency distribution observed in Nouville1 for *Mimachlamys gloriosa* compared with the other species and sites. Douchement and Chauvet (1994) and this study both reported size frequency distribution for *Mimachlamys gloriosa* juveniles of more than 7 mm was unimodal with a left skewness.

Growth of *Bractechlamys vexillum*

No significant links were observed between tray position and growth of *Bractechlamys vexillum*. The significant differences of K values observed in the high-density lanterns were probably due to variable individual growth and mortality within the trays. Conversely, growth was probably linked to density. K values were not statistically different but size at the end of the rearing period and H_{∞} values were higher in the low-density lanterns. Parsons and Dadswell (1992) found an inverse relationship between growth and stocking density for a temperate scallop species (*Placopecten magellanicus*), and Maeda-Martinez et al. (1997) observed lower growth rates at the highest densities at each of the three culture stages of a tropical scallop (*Argopecten ventricosus*). Limited trophic resources in the high-density lanterns when specimens attain larger size, could explain these differences during the second part of the rearing period (after 200 rearing days). Site had a significant effect on growth. K was lower but specimens probably reached larger sizes in Baie des Citrons2. It is possible that a higher productivity at this site (coastal bay where terrestrial runoff may be important) minimized the impact of limited trophic resources. It was also observed during maintenance operations that fouling was more abundant and developed more quickly in Baie des Citrons2 than Nouville2. Parameters of the Von bertalanffy growth model in experimental rearing structures ($K > 0.07$ per week and $H_{\infty} < 42$ mm) were characteristic of a higher initial growth rate but a lower maximum size than the estimations of Lefort and Clavier (1992) for natural populations in the same lagoon ($K = 0.036$ per week and $H_{\infty} = 47.4$ mm). Two factors could explain why growth in artificial structures is not optimal: limitation of trophic resources and a mortality affecting the bigger specimens at the end of the rearing period. The influence of fouling on growth is known to be significant (Lodeiros & Himmelman 2000). This effect should be limited during this study because fouling was regularly removed. Size differences between specimens after 12 and 18 months were very low (<2 mm) in all the lanterns.

Growth of *Mimachlamys gloriosa*

Tray position, density and site had no significant effect on K parameter of Von bertalanffy growth model for *Mimachlamys gloriosa*. However, density probably had a limited effect on growth because the specimens reached a larger size and H_{∞} was higher in the low-density lanterns. Parameters of the Von bertalanffy growth model in experimental rearing structures ($K = 0.066$ and $H_{\infty} = 57$ mm) were characteristic of a higher initial growth rate but a lower maximum size than the estimations of Lefort and Clavier (1992) for natural populations in the same lagoon ($K = 0.019$ per week and $H_{\infty} = 73.9$ mm). The growth of this species was probably not optimal in the artificial rearing structure at the densities tested. The duration of the rearing phase was sufficient for specimens to reach the maximum sizes observed in natural populations. However, the

lower mean size reached in experimental conditions was probably due to limited trophic resources in both sites during the second part of the rearing period. Because *Mimachlamys gloriosa* is a larger species than *Bractechlamys vexillum* it probably had higher feeding demand. Therefore, space between trays and water circulation in the lantern may have been too limited when specimens reached a certain size. Moreover, competitive sponges colonized the pectinids' shell. Another significant factor affecting maximum size is mortality, which affected the larger specimens at the end of the rearing period. Size differences between specimens after 12 and 18 months were very low (< 2 mm) in all the lanterns.

Mortality of *Bractechlamys vexillum*

Mortality of *Bractechlamys vexillum* in the rearing structures was highly variable. The high mortality rates after the transfer of juveniles were probably related to biologic stress during handling procedures and death of smaller specimens trapped between the tray structures. Well-developed gonads were observed after 150–250 rearing days corresponding to sexual maturity (Lefort & Clavier 1992). This period was also characterized by punctual high mortality rates related to the first reproduction event, particularly in Baie des Citrons². Lodeiros and Himmelman (2000) reported a decreased survival in the period during and just after spawning for a tropical scallop (*Envula ziczac*), possibly coincident with physiologic stress from spawning. After reaching sexual maturity this species is characterized by continuous reproduction. Therefore, the impact on mortality was not detectable. Other punctual mortality events were related to predation by mollusks (Cymatiidae) or crabs (*Portunus*) in some trays and the possible effect of pathogens. The significant mortality at the end of the rearing period was probably due to the senescence of the population. Density had a significant effect on the mortality of *Bractechlamys vexillum*. A higher mortality (Z and cumulated mortality) occurred in the low-density lanterns at each site. Site only had a significant effect on high-density lanterns. The action of pathogens in low-density lanterns, highly variable density results, densities tested below a threshold in which significant differences would be detected, or the action of other uncontrolled factors could explain this unexpected result. However it was not possible to identify these factors from the results of this study.

Z values calculated during this study ($Z = 0.16\text{--}0.43$ per year) were lower than the values calculated by Lefort (1991) and Lefort and Clavier (1992) (0.86 per year) for natural populations in the same lagoon. This result is consistent because of the limited predation in artificial rearing conditions, because a net protects specimens. The only predators found in the lanterns during the rearing phase were mollusks (*Septa tabulata*) and Portunid crabs (*Portunus*). These predators recruited in the lanterns and had begun to grow before being removed during maintenance operations.

Mortality of *Mimachlamys gloriosa*

Mortality rates were variable and Z coefficients were significantly different between trays. Z was higher in trays located at the bottom of the lanterns, in particular in the Baie des Citrons² high-density lantern. However, mortality rates displayed no gradient from top to bottom. *Mimachlamys gloriosa*, unlike *Bractechlamys vexillum*, was not affected by high mortality rates after the transfer of juveniles. The transferred juveniles were larger and did not get trapped between trays. Moreover, this species is byssally attached which may have contributed to lower mortality by avoiding en-

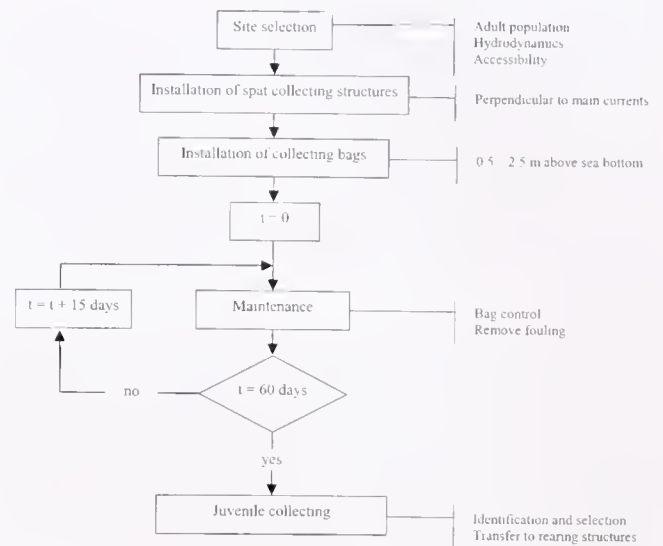


Figure 10. Spat collecting protocol for *Bractechlamys vexillum* and *Mimachlamys gloriosa*

trapment and imbrications. The mortality probably increased at the end of the rearing period because of the senescence of the population. Density and site had significant effects on the mortality of *Mimachlamys gloriosa*. Mortality was higher in the high-density lanterns, this pattern being the opposite of *Bractechlamys vexillum*. Competition for food and space could explain this result. However, Parsons and Dadswell (1992) found that survival of a temperate scallop species (*Placopecten magellanicus*) was not influenced by density. When the specimens grow and reach sexual maturity, food resources can become too low at a time when more energy is necessary for reproduction. Lodeiros and Himmelman (2000) reported a decreased survival in the period during and just after spawning for a tropical scallop (*Envula ziczac*), possibly coincident with physiologic stress from spawning and low phytoplankton abundance and high temperatures. Consequently, specimens weaken and become more sensitive to pathogens and toxic phytoplankton. This phenomenon was more significant in Baie des

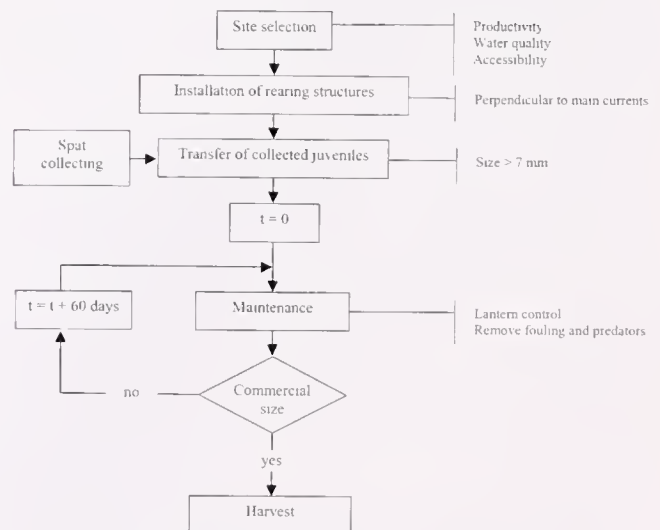


Figure 11. Optimized juvenile rearing protocol for *Bractechlamys vexillum* and *Mimachlamys gloriosa*.

Citrons2, where anthropogenic impact is more important (sewage and beach) and phytoplankton blooms were observed.

Z values calculated for this study ($Z = 0.16\text{--}0.55$ per year) were close to those given by Lefort (1991) and Lefort and Clavier (1992) (0.42 per year). However, these values were lower in all lanterns, except that at Baie des Citrons2. Predation was reduced in the rearing structure because of the protection of the net and the removal of predators during maintenance procedures.

Aquaculture protocol optimization

The spat-collecting phase was successful and a protocol can be proposed (Fig. 10). Adult populations should be present at the collecting site to optimize collecting yields. The rearing phase was also successful but could be optimized. Both species reached a commercial size within 12 months. Growth rates decreased and mortality rates increased after 12 months. With a rearing phase limited to 12 months the size of *Bractechlamys vexillum* should vary between 41.9 and 44.5 mm depending on density versus 42.2

to 45.3 mm for an 18-month rearing period. However, survivorship would increase to 66% to 85% (12 mo period) versus 55% to 80% (18 mo period). For *Mimachlamys gloriosa* size would decrease from 56.3 mm (18 mo period) to 54.8 mm (12 mo period) on average but survivorship should increase to 58% to 86% (12 mo period) versus 46% to 80% (18 mo period) depending on density. The rearing site must be located in a productive area where sea-water quality is suitable for the species. High-density lanterns bring better yields than low densities and should be preferred for a 12-month rearing period. An optimized rearing protocol is presented in Figure 11.

ACKNOWLEDGMENT

The authors thank Richard Farman and François Devineck for supporting this study and the crews of the Southern Province patrol boats, particularly Gérard Charmean, Michel Blanc and Christophe Gosset, for their technical assistance during the survey. The authors thank the anonymous referees for constructive comments on the manuscript.

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EFFECT OF DEPTH AND STOCKING DENSITY ON GROWTH AND RETRIEVAL OF THE POSTLARVAL LION'S PAW SCALLOP, *NODIPECTEN NODOSUS* (LINNAEUS, 1758)

GUILHERME S. RUPP,^{1,*} G. JAY PARSONS,^{2,†} RAYMOND J. THOMPSON¹ AND MICHELINE M. DE BEM³

¹Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, NL, Canada A1C 5S7;

²Fisheries and Marine Institute, Memorial University of Newfoundland, St. John's, NL, Canada A1C

5R3; and ³Laboratório de Cultivo de Moluscos Marinhos, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil 88062-601

ABSTRACT Coastal areas of southern Brazil are experiencing an intense development of bivalve farming, and the lion's paw scallop, *Nodipecten nodosus* (Linnaeus, 1758), is a promising aquaculture species. An important but poorly understood oceanographic feature described for the inner continental shelf of the southeastern Brazilian coast is the seasonal subsurface intrusion of cold and phytoplankton-rich South Atlantic Central Water (SACW) during summer–autumn. It was not clear, however, if these intrusions could affect near-shore coastal areas off Santa Catarina State, where aquaculture activities are now expanding, nor if they could affect growth and survival of bivalves under culture conditions. It was therefore hypothesized that intrusions of SACW with higher food levels and lower temperature would result in depth-related differences in environmental factors that would affect bivalve growth. One experiment was carried out in summer–early autumn (March–April 2001), a period of possible SACW intrusion in a coastal site, and a second experiment was carried out in late autumn–early winter (June–July 2001) when the water column would possibly be homogeneous. In both experiments, hatchery-produced postlarval *N. nodosus* (0.4 mm) were deployed in the sea-based nursery 12–15 days after settlement and retrieved 26–27 days later. Postlarvae were attached to Netlon collectors, placed inside nylon bags, and hung on a subsurface long-line at 4 and 12 m. The effects of deploying postlarvae at two densities (340 and 150 scallops/spat bag) were also investigated. Subsurface intrusions of cold and phytoplankton-rich SACW occurred at a near-shore (<0.5 km) coastal site in late summer–early autumn but not in late autumn–early winter. The influence of SACW resulted in oscillating temperatures (17.8–27.2°C) on a short time-scale and in high particulate inorganic matter (PIM), which reduced postlarval growth at 12 m compared with 4 m, but did not affect survival. When SACW was not present, there were no depth-related differences. Postlarvae cultured at high density displayed slightly smaller shell heights at both depths than postlarvae kept at low density but displayed similar survival, resulting in higher yields (no. spat/bag). The use of a subsurface long-line for deploying hatchery-produced postlarvae (0.4 mm) resulted in percentage retrievals of about 70–80%, regardless of treatment and season, values that are higher than in previous studies. There is a good potential for growing *N. nodosus* in sea-based nurseries in coastal areas of southern Brazil, and growth can be optimized by seasonally adjusting culture depth.

KEY WORDS: environmental factors, growth, *Nodipecten nodosus*, nursery culture, postlarvae, scallop, survival

INTRODUCTION

Coastal sheltered areas off Santa Catarina State, in southern Brazil, are currently experiencing an intense development of bivalve aquaculture, but the influence of environmental variability on bivalve growth and survival has only recently been addressed (Rupp 2003). As coastal embayments continue to be used, there will be a need in the near future to expand aquaculture activities to deeper areas farther from shore. It is therefore crucial to understand the influence of depth-related variability in environmental factors on growth and survivorship of bivalves to establish sustainable culture strategies. The lion's paw scallop, *Nodipecten nodosus* (Linnaeus, 1758), is a promising aquaculture species in Brazil, and an understanding of the influence of environmental factors on growth and survival are essential for the establishment of this new activity.

The South Brazil Bight (SBB) between Cabo Frio (23°S) and Cabo de Santa Marta (28.5°S) is one of the most economically and ecologically important regions on the Brazilian coast, but the effects of its oceanographic variability are still poorly understood (Brandini 1990, Campos et al. 1996, Borzone et al. 1999), and no previous studies have assessed the influence of spatial variability

of environmental factors on bivalve growth. An important and poorly understood oceanographic feature influencing the SBB is the seasonal subsurface intrusion of cold and phytoplankton-rich South Atlantic Central Water (SACW), which ascends the continental slope from deeper layers, influencing the shelf region (Matsuura 1986, Brandini 1990, Campos et al. 1996, Castro & Miranda 1998, Sunyé & Servain 1998). Except for coastal upwelling at the extreme limits of the SBB, SACW has previously been considered to influence mainly the middle and outer continental shelf, detected at distances greater than 30 km from shore (Castro & Miranda 1998). However, subsurface intrusions of SACW can influence inner shelf waters up to 15 m in a 38-m-deep station during summer (Borzone et al. 1999). This oceanographic feature may have an important influence on growth and reproduction of benthic invertebrates, but to date biological effects of SACW on marine bivalves have not been addressed. Furthermore, it is not known whether SACW can reach near-shore (<1 km) coastal areas off Santa Catarina State where aquaculture activities are now expanding, nor if these subsurface intrusions could affect growth and survival of cultured bivalves to such a degree as to be an important factor to be considered when establishing aquaculture sites and husbandry practices.

Whereas the influence of depth on growth of juvenile and adult scallops has extensively been studied in temperate and boreal regions (examples in Bricelj & Shumway 1991, Thompson & Mac-

*Corresponding author. E-mail rupp@epagri.rct-sc.br

Donald 1991), none of these studies were carried out in a subtropical environment, nor have they focused on the postlarval stage. Though some studies have reported slower growth near the surface (MacDonald & Bourne 1989, Román et al. 1999), growth usually decreases below a critical depth, as environmental conditions such as temperature, food availability, or turbidity often display a vertical gradient, reaching suboptimal levels in deeper waters (Leighton 1979, MacDonald & Thompson 1985, MacDonald & Bourne 1989, Côté et al. 1993, Emerson et al. 1994, Lodeiros et al. 1998, Grecian et al. 2000, Lodeiros & Himmelman 2000, Fréchette & Daigle 2002). In view of the environmental variability characteristic of waters off southern Brazil, it is possible that an unusual situation of high food level and low temperature below a crucial depth could influence growth of scallops in suspended culture close to shore.

The stocking density of bivalves within enclosures is an important variable to be considered in aquaculture, due to intraspecific competition for food and space. Density-dependent growth of juvenile and adult scallops cultured in lantern or pearl nets has been well documented (Duggan 1973, Ventilla 1982, Parsons & Dadswell 1992, Côté et al. 1993, Côté et al. 1994, Maeda-Martinez et al. 1997, Román et al. 1999), but few studies have focused on postlarval scallops within the size range examined in the current work, in which spat were attached to the collectors by byssal threads instead of lying on the floor of the culture nets (Grecian et al. 2000).

The main objective of the current study was to investigate the influences of environmental factors associated with depth on growth and percentage retrievals of postlarval *N. nodosus*. Experiments were carried out during a period when SACW could possibly influence the coastal site (experiment 1) (March–April) as well as when no influence of SACW was expected (experiment 2) (June–July). It was hypothesized that a cold and phytoplankton-rich water mass originating from SACW intrusions could reach the coastal aquaculture site in late summer, resulting in significant depth-related differences in growth of postlarval scallops above and below the thermocline. Conversely, in periods when the SACW is absent, growth should be similar at different depths. A second objective was to investigate the effects of stocking density on growth of postlarval scallops.

MATERIALS AND METHODS

Study Area

Experiments were undertaken off Porto Belo Point (Lat. 27°07'S; Long. 48°30.8'W), Santa Catarina State, Brazil (Fig. 1). A subsurface long-line, on which the main line was located 3 m below the surface, was deployed at a 15-m-deep site, 150 m from shore.

Spat Production

Experimental production of postlarval *N. nodosus* was undertaken at the Laboratory for the Culture of Marine Molluscs (LCMM-UFSC), Florianópolis, Santa Catarina State, Brazil. Two experimental spat productions were carried out in February and May 2001, using procedures described in Rupp et al. (2004). When larvae were competent to undergo settlement and metamorphosis, they were retained on a 140- μ m-mesh nytex screen, counted, and transferred to the experimental tanks. Settlement occurred in 200-L tanks, with pediveligers stocked at densities of 1.2 larvae mL⁻¹.

Japanese-type Netlon monofilament collectors of about 1.5-m long (averaging 105.2 g, SE = 1.2; n = 9), commonly used in scallop commercial operations, were used as substrate for settlement. During settlement, seawater (salinity = 33‰ to 34‰) was UV-irradiated and passed through a 1- μ m cartridge filter. Water was changed daily, and a food ration consisting of a mixture of *Isochrysis galbana* (T-iso), *Chaetoceros calcitrans*, and *C. muelleri* (2:2:1) in exponential growth phase was supplied daily in final concentrations ranging from 4×10^4 to 5×10^4 cells mL⁻¹. In the laboratory, water temperatures were 24–25°C and 21–22°C for experiments 1 and 2, respectively.

Experimental Approach

In experiment 1, carried out from mid-March to mid-April 2001 (late summer–early autumn), postlarval scallops were transferred to the field 12 days after settlement. The Netlon collectors were taken from the setting tanks, placed inside 1.5-mm-mesh nylon spat bags (1 collector/bag), and then transferred to the sea. The nylon spat bags were inserted into plastic bags filled with filtered seawater that were tightly closed and transported to the field site inside Styrofoam containers. Prior to deployment, four collectors per tank were sampled to estimate the initial number and size of postlarvae. Collectors were thoroughly brushed with repeated gentle movements inside plastic trays filled with filtered seawater. Detached spat were then screened, separated from debris, and preserved in 4% buffered formaldehyde for further counting and measurements. Initial sampling indicated two different densities of spat in different setting tanks, which are hereafter termed “high” (340 scallops/collector, SE = 54.7) and “low” (150 scallops/collector, SE = 47.0) densities. Deployment was undertaken by scuba diving, spat bags being tied at depths of 4 and 12 m from the surface to two ropes hanging perpendicularly from the long-line. A 2-kg weight was attached to the bottom of each drop line. Spat bags (high and low densities) were tied individually to the ropes at 4 and 12 m. In this manner, a factorial design with two replicates was used to evaluate the effects of depth and density on growth of postlarval scallops. Control collectors were deployed in the sea simultaneously to the experimental units to monitor wild scallop settlement. All collectors were retrieved 26 days after deployment (38 days post-set) and transferred to the laboratory.

In experiment 2, carried out from early June to early July 2001 (late autumn–early winter), postlarval scallops were transferred to the field 15 days after settlement. The collectors, spat bags, and procedures were similar to experiment 1. As initial sampling indicated that numbers of spat/collector were similar among setting tanks (401 scallops/collector, SE = 95.9), single spat-bags (1 collector/bag) were tied to triplicate ropes hanging perpendicularly to the long-line at 4 and 12 m depth. Collectors were retrieved 27 days after deployment (42 days post-set) and transferred to the laboratory, where spat were detached and sampled as previously described.

For both experiments, the initial shell height before deployment (maximum dorso-ventral distance perpendicular to the umbo) (n = 30) and final shell height at retrieval (n = 30/spat bag) were determined with an ocular micrometer. To estimate the degree of clogging of the spat bags, individual bags from both experiments were thoroughly scrubbed inside plastic containers after retrieval. The sediments and fouling material deposited in the containers were collected on preweighed 0.5 m \times 0.5 m cellulose filter papers folded into a funnel shape. The filters were then dried for 72 h at 60°C, cooled in a desiccator, and reweighed to determine the dry



Figure 1. Location of the study site off Porto Belo Point (*), (Lat. 27°07'S; Long. 48°30.8'W), Porto Belo, Santa Catarina State (SC), Brazil.

weight of the sediments and fouling material attached to the external spat bags.

Environmental Variables

Water samples from 4- and 12-m depths were taken weekly at the field site using a 2-L Van Dorn sampling bottle. Samples were transferred to the laboratory in a cooler containing ice packs. Temperatures at both depths were recorded hourly with Stowaway data loggers, and dissolved oxygen was determined *in situ* with a digital dissolved oxygen meter (YSI model 55/12FT). In the laboratory,

water samples were gently stirred and filtered through a 350- μ m-mesh screen to remove larger zooplankton. Total suspended particulate matter was determined gravimetrically after filtering 400 mL seawater through a 24-mm-diameter Whatman 934-AH glass microfiber filter (nominal pore size 1.5 μ m) and drying for 72 h at 60°C. Particulate organic (POM) and inorganic matter (PIM) were determined by weight loss on ignition at 450°C (Parsons & Dadswell 1992). Chlorophyll-*a*, an index of phytoplankton biomass, was determined with a Turner digital fluorometer (TD 10-AU) after filtering triplicate, 100-mL water samples (Whatman 934-

AH) and extracting chloropigments in 90% acetone using standard methods (Method. 445.0, USEPA 1997). Salinity was determined by a digital WTW Multiline P4 water quality meter. All variables were determined in triplicate samples.

Statistical Analyses

Data were analyzed with the SPSS statistical package (version 10). Residuals were checked graphically for normality and homoscedasticity (Sokal & Rohlf 1995). A factorial design was used in experiment 1, and a two-way ANOVA ($\alpha = 0.05$) was performed to test for differences in shell heights and percentage retrievals at different depths and densities, using depth and density as fixed factors. In experiment 2, an independent sample *t*-test was used to determine the differences in shell height and percentage retrieval between depths.

RESULTS

Growth

In experiment 1, carried out from mid-March to mid-April 2001, postlarval scallops were deployed in the sea at an initial mean shell height of 0.40 mm. After 26 days of immersion, the mean shell height of scallops at 4 m depth was 5.07 mm (SE = 0.13) and 5.67 mm (SE = 0.16) for collectors with high and low densities, respectively (Fig. 2A), whereas at 12 m the corresponding shell heights were 3.28 mm (SE = 0.10) and 3.60 mm (SE = 0.11). Final shell height was significantly greater at 4 m than at 12 m (ANOVA, $F = 232.4$; $df = 1, 236$; $P < 0.001$) and also greater at low stocking density than at high density (ANOVA, $F = 13.31$; $df = 1, 236$; $P < 0.001$). The interaction between density and depth was not significant ($P = 0.26$). The resulting daily growth rates (DGR) at 4 m were $0.179 \text{ mm day}^{-1}$ and $0.203 \text{ mm day}^{-1}$ for scallops grown at high and low densities, respectively, whereas at

12 m, DGR was $0.110 \text{ mm day}^{-1}$ and $0.123 \text{ mm day}^{-1}$, respectively.

In experiment 2, carried out from early June to early July 2001, postlarval scallops were deployed in the sea at an initial mean shell height of 0.43 mm. No significant difference in mean shell height was detected between depths (*t* test, $t = 1.93$; $df = 1, 178$; $P = 0.056$). Mean shell height at retrieval (after 27 days) was 4.34 mm (SE = 0.25) at 4 m and 4.12 mm (SE = 0.15) at 12 m (Fig. 2B). DGR was 0.144 and $0.137 \text{ mm day}^{-1}$ at 4 and 12 m, respectively.

The dry weight of sediments attached to the spat bags on retrieval was 33.05 g (SE = 1.47) and 30.06 g (SE = 2.16) for 4 m and 12 m, respectively, in experiment 1 and 35.01 g (SE = 1.55) and 34.18 g (SE = 5.12) for 4 m and 12 m, respectively, in experiment 2. There was no significant difference in the sediment content between depths in both experiments, eliminating sediment load as a factor influencing growth rate (*t*-test, $t = 1.5$, $df = 1, 4$; $P = 0.10$ in experiment 1 and $t = 0.99$, $df = 1, 6$; $P = 0.18$ in experiment 2). No wild spat of *N. nodosus* settled on the control collectors in each experiment.

Percentage Retrievals

In experiment 1, the mean number of spat retrieved from the collectors at 4 m was 247 (SE = 42) and 111 (SE = 10) for high and low densities, respectively, whereas at 12 m, the number of spat per collector was 245 (SE = 87) and 122 (SE = 2), respectively. The percentage retrievals were similar between depths and densities (ANOVA, $F = 0.05$; $df = 3, 4$; $P = 0.9$), ranging between 72.2% and 81.3% (Fig. 3A). In experiment 2, the mean number of spat retrieved per collector was 317 (SE = 28) and 278 (SE = 54) at 4 m and 12 m, respectively. Percentage retrievals

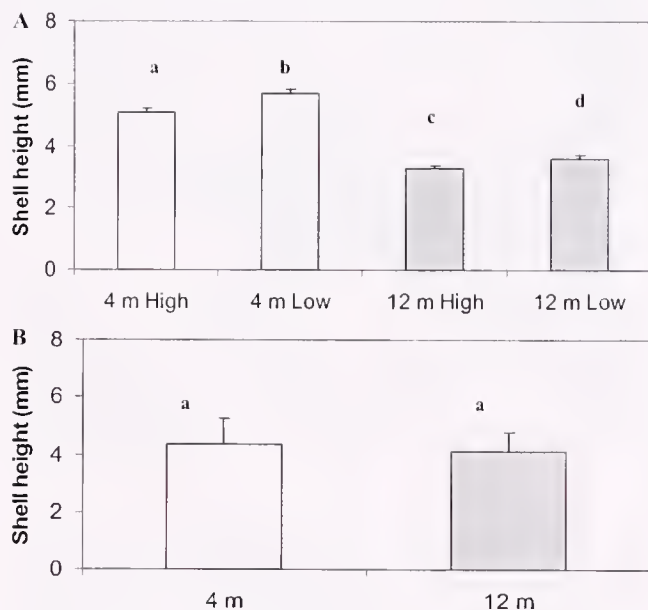


Figure 2. (A) Final shell heights (mm) of scallops cultured at 4 m and 12 m depth at high (350 spat/collector) and low (140 spat/collector) stocking densities for 26 days off Porto Belo Point, experiment 1 (March–April 2001). (B) Final shell heights (mm) of scallops cultured at 4 m and 12 m depth for 27 days off Porto Belo Point, experiment 2 (June–July 2001) (common letters denote no significant differences; vertical bars = \pm SE).

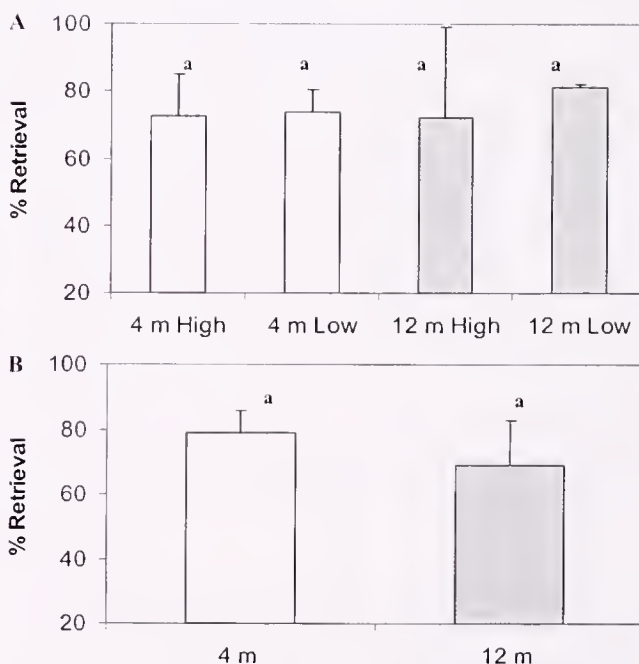


Figure 3. (A) Percentage retrieval of scallops cultured at 4 m and 12 m depth at high (350 spat/collector) and low (140 spat/collector) stocking densities for 26 days off Porto Belo Point, experiment 1 (March–April 2001). (B) Percentage retrieval of scallops cultured at 4 m and 12 m depth for 27 days off Porto Belo Point, experiment 2 (June–July 2001) (common letters denote no significant differences; vertical bar = \pm SE mean \pm SE).

were similar between depths (*t*-test, $t = 0.64$; $df = 4$; $P = 0.56$), ranging from 69.3% to 79.05% (Fig. 3B).

Environmental Variables

Temperature

Water temperature in experiment 1 (March–April), recorded hourly, was highly variable (Fig. 4A), especially in the first 2–3 wk of the experiment (March), after which the water column became homogeneous (April). At 12 m, temperature variability (CV = 10.4%) was greater than at 4 m (CV = 5.3%) and temperatures were lower. The mean temperature at 4 m was 25.3°C, with a maximum of 28.2°C and a minimum of 19.6°C. At 12 m, mean temperature was 22.8°C, with a maximum of 27.2°C and a minimum of 17.8°C. On several occasions in March, temperature at 12 m varied by 6–9°C within a 24 h period.

In experiment 2, from early June–early July, temperature was uniform throughout the water column and thus similar at both depths (Fig. 4B). The mean temperature was 20.4°C and 20.1°C at 4 and 12 m, respectively. Whereas maximum temperature at 4 m and 12 m was 22.2°C and 21.7°C, respectively, the minimum temperature at both depths was 17.8°C.

Salinity

There was negligible variation in salinity at 12 m (mean = 33.3%) throughout experiment 1 (Fig. 5A), whereas at 4 m mean salinity was 32.2%, and a lower value (29%) was recorded on one occasion. In experiment 2, the water column was homogeneous with mean salinities of 32.1% and 32.4% at 4 m and 12 m, respectively.

Chlorophyll-*a*

The concentration of chlorophyll-*a* was highly variable during experiment 1, and there were marked differences between 4 and 12 m (Fig. 5B). The mean concentration at 12 m ($1.16 \mu\text{g L}^{-1}$) was

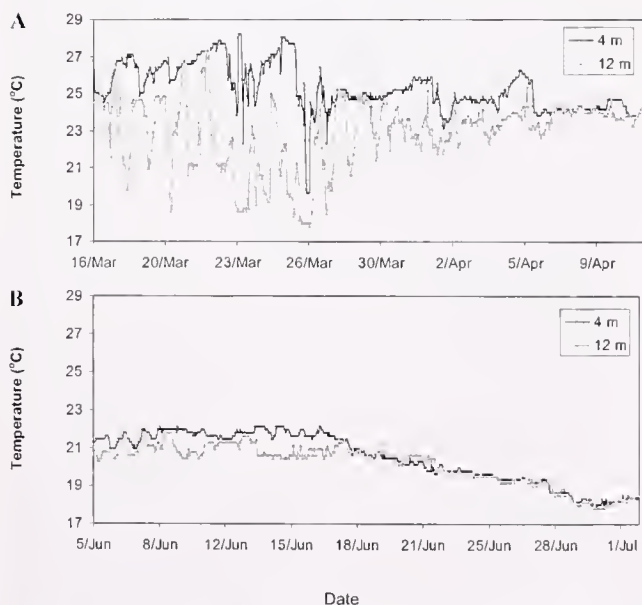


Figure 4. Hourly temperature at 4 m and 12 m depth off Porto Belo Point: (A) experiment 1 (March–April 2001); (B) experiment 2 (June–July 2001).

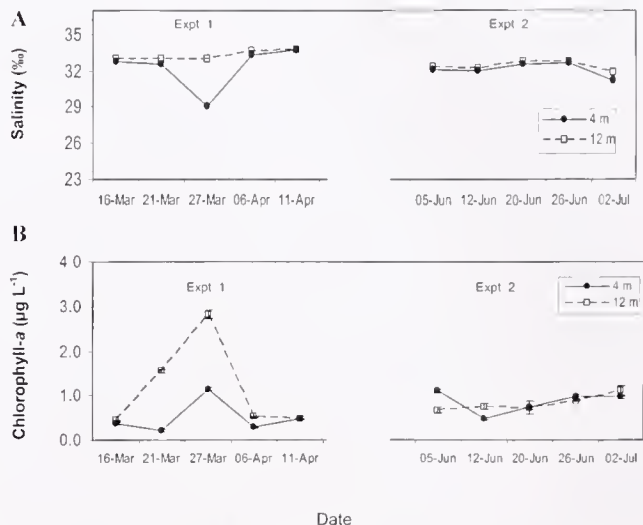


Figure 5. (A) Salinity (‰) and (B) chlorophyll-*a* ($\mu\text{g L}^{-1}$) at 4 m and 12 m depth off Porto Belo Point during experiments 1 (March–April 2001) and 2 (June–July 2001) (mean \pm SE; where bars not visible, SE is too small to plot).

more than twice that at 4 m ($0.5 \mu\text{g L}^{-1}$). Maximum concentrations at 12 m reached 1.55 and $2.82 \mu\text{g L}^{-1}$ in samplings 2 and 3, respectively, which coincided with periods of sharp differences in temperature between 4 and 12 m. At 4 m, mean concentrations were $0.21 \mu\text{g L}^{-1}$ and $1.15 \mu\text{g L}^{-1}$, respectively, for these samplings. In experiment 2, on the other hand, variability in chlorophyll-*a* was relatively small and there were negligible differences between depths. At 4 m the mean value of chlorophyll-*a* was $0.85 \mu\text{g L}^{-1}$, whereas at 12 m it was $0.82 \mu\text{g L}^{-1}$.

Seston

Total particulate matter (TPM) was highly variable in experiment 1 and was higher at 12 m (mean = 2.17 mg L^{-1}) than at 4 m (mean = 1.16 mg L^{-1}) (Fig. 6A), whereas in experiment 2 there were negligible differences in TPM between 4 m (mean = 1.56 mg L^{-1}) and 12 m (mean = 1.71 mg L^{-1}), and TPM was higher toward the end of the experiment.

In experiment 1, particulate inorganic matter (PIM) was higher at 12 m, averaging 1.76 mg L^{-1} , whereas at 4 m, mean PIM was 0.69 mg L^{-1} (Fig. 6B). Particulate organic matter (POM) followed a similar pattern at both depths with a mean of 0.47 mg L^{-1} at 4 m and 0.44 mg L^{-1} at 12 m (Fig. 6C). PIM as percentage of TPM was higher at 12 m (mean = 81.4%) than at 4 m (mean = 59.6%) (Fig. 7A). In contrast, in experiment 2, PIM values were similar at 4 and 12 m, averaging 1.10 mg L^{-1} and 1.14 mg L^{-1} , respectively (Fig. 6B). Differences in POM levels between 4 m (mean = 0.46 mg L^{-1}) and 12 m (mean = 0.57 mg L^{-1}) were negligible (Fig. 6C). As a result, mean PIM as a percentage of TPM was 69.7% and 65.2% at 4 and 12 m, respectively (Fig. 7A).

Dissolved Oxygen

In experiment 1, dissolved oxygen was higher at 4 m, where percent saturation ranged from 82.8% to 92.1% (mean = 91.1%) (Fig. 7B), than at 12 m (mean = 75.3%), where minimum levels reached 61.9% and 48.8%. These dissolved oxygen minima coincided with periods when the temperature at 12 m was about 6°C lower than at 4 m. In experiment 2, mean percent oxygen saturation was 90.82% and 84.50% at 4 m and 12 m, respectively.

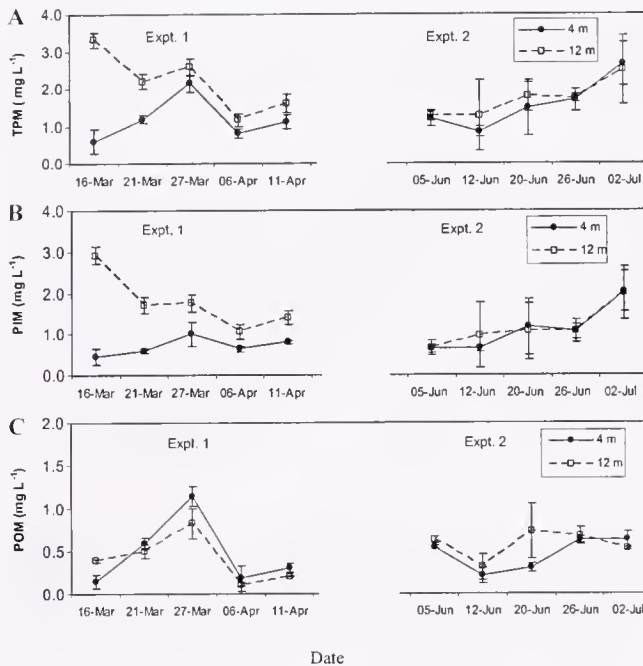


Figure 6. (A) Total particulate matter (TPM), (B) particulate inorganic matter (PIM), and (C) particulate organic matter (POM) (mg L^{-1}) at 4 m and 12 m depth off Porto Belo Point during experiments 1 (March–April 2001) and 2 (June–July 2001) (mean \pm SD).

DISCUSSION

Growth

Differences in growth of postlarval *Nodipecten nodosus* were recorded between 4 and 12 m in experiment 1 during late summer–early autumn. Scallops attained a larger size near the surface, where the growth rate was approximately 1.6 times higher than at 12 m. However, in experiment 2 (late autumn–early winter), growth of scallops was similar at both depths. Whereas similar growth rates in experiment 2 may be explained by the homoge-

neous conditions throughout the water column, the differential growth recorded in experiment 1 may be attributed to differences in environmental factors between depths.

This is the first study to report the influence of a subsurface intrusion of cold water with higher phytoplankton biomass in a coastal aquaculture area in southern Brazil and to demonstrate its significant effect on bivalve farming. Although food quantity, as expressed by concentration of chlorophyll-*a*, was higher at 12 m in experiment 1, growth of postlarval scallops was lower. Differences in mean temperature between depths were probably too small (2.5°C) to account for the observed differences in growth. However, the higher temperature variation on a short time-scale recorded at 12 m, together with the longer exposure period to lower temperatures, may have stressed the scallops, thus limiting growth. In addition, whereas the temperature dropped below 20°C at 4 m for only 4 h, at 12 m this persisted for at least 78 h, a period in which growth rates could have been reduced. Growth in bivalves is an integrated response to food availability and temperature (Bayne & Newell 1983, MacDonald & Thompson 1985, Bricelj & Shumway 1991, Parsons & Dadswell 1992, Côté et al. 1993). No studies on bivalve growth have considered the effects of temperature variation on such a short time-scale as in the current study (ca. semi-daily). In a study by Pilditch & Grant (1999), temperature variation on an 8-day cycle had a negligible effect on growth and metabolism of juvenile scallops *Placopecten magellanicus* under laboratory conditions. Scallops did not acclimate to the temperature cycles, displaying metabolic rates tightly coupled with water temperature, which is also in agreement with Shumway et al. (1988), who found that metabolic rates of *P. magellanicus* follow the seasonal and laboratory acclimation temperatures. In Newfoundland, feeding and metabolic rates of *P. magellanicus* are also correlated with environmental temperature, as well as food availability (MacDonald & Thompson 1986). In contrast, Widdows (1976) demonstrated compensatory acclimation to temperature in the mussel (*Mytilus edulis*). A strong dependence of physiologic rates on environmental temperature in scallops suggests that the periods of lower temperature may have depressed physiologic rates in *N. nodosus*, contributing to the observed reduction in growth during periods of lower temperature.

PIM as a percentage of TPM is an important environmental factor affecting growth of bivalves. The amount of PIM in seawater dilutes POM, reducing the nutritional value of seston for scallops (Vahl 1980, Wallace & Reinsnes 1985, Bricelj & Shumway 1991, Emerson et al. 1994). For example, when PIM reaches a critical value of about 80% of TPM, *Chlamys islandica* cannot absorb POM, resulting in a reduction of feeding rate, thus reducing or stopping growth (Vahl 1980). Although certain bivalves (including some scallops) can selectively ingest organic particles to compensate for dilution of food quality (Widdows et al. 1979, Bricelj & Malouf 1984, MacDonald & Ward 1994), *P. magellanicus* lacks this ability at high PIM concentrations (Bacon et al. 1998). In experiment 1, percent PIM was markedly higher at 12 m than at 4 m, averaging 81.4% and reaching a maximum of 90.5%. Thus, the benefits of higher food abundance, as expressed by levels of chlorophyll-*a*, were probably offset by the negative effect of a higher inorganic content of seston, resulting in significantly lower growth rates at 12 m. It is therefore difficult to decouple the effects of temperature and inorganic content of the seston when determining which was the major factor affecting growth, as high PIM was associated with temperature oscillations in experiment 1. It is more likely, however, that an interaction of both variables

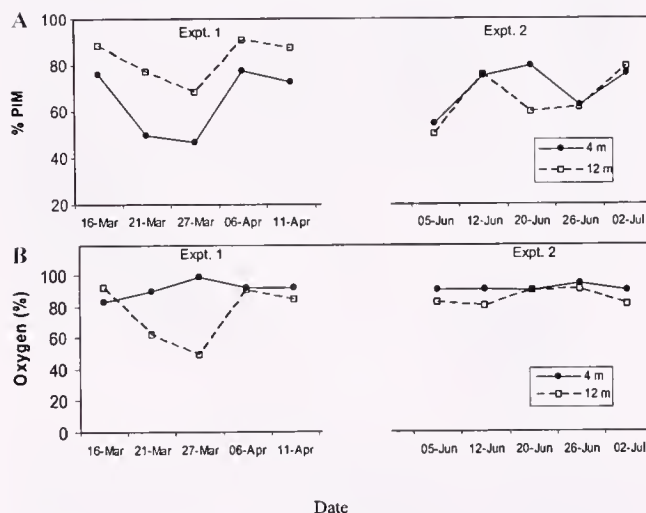


Figure 7. (A) Percentage of inorganic matter in relation to total seston (% PIM) and (B) percent oxygen saturation at 4 m and 12 m depth off Porto Belo Point during experiments 1 (March–April 2001) and 2 (June–July 2001).

contributed to the slower growth recorded at 12 m. In general, POM concentrations were similar between depths, but on one occasion an unusual situation arose in which POM was higher at 4 m, but chlorophyll-*a* was higher at 12 m. This coincided with a low salinity event at 4 m, probably caused by continental runoff due to rainfall, which may have exported terrigenous organic matter.

The food supply for cultured bivalves is also largely influenced by water flow and the concentration, size, and quality of food particles (Grant 1996). It is unlikely that water flow differed between 4 and 12 m in the current study, causing differences in growth, because such differences would have also been observed in experiment 2, in which growth was similar between depths. The size and quality of the suspended particles removed by bivalves is dependent on the phytoplankton species composition, which was not evaluated in the current study. The possibility that phytoplankton composition differed between depths in experiment 1 cannot therefore be ruled out and requires further investigation. Although several studies have examined the phytoplankton composition in coastal and oceanic regions of southern Brazil (citations in Soares 1983), none have focused on the phytoplankton composition of subsurface intrusions of SACW. Nevertheless, from an aquaculture perspective, it is significant that during summer-early autumn, the coastal culture site is under the influence of a thermocline oscillation caused by the proximity of SACW, and under these circumstances, nutritional and thermal conditions are less favorable for growth of postlarval scallops.

Brandini et al. (2000) proposed that if bivalves were grown offshore in southern Brazil in depths influenced by SACW, growth would be maximized due to higher food abundance as expressed by levels of chlorophyll-*a*. The current study suggests that growing scallops below the thermocline during the SACW intrusion may not necessarily be beneficial, as other concurrent factors may neutralize the benefits of enhanced food levels. It is possible, however, that the influence of SACW on growth may vary according to the species under consideration. *Nodipecten nodosus* occurs in tropical waters (Smith 1991), and the study area is at its southern limit of distribution in the Southern Hemisphere, where minimum temperatures reach levels reducing growth rates (Rupp 2003). Furthermore, scallops display low tolerance for high PIM (examples in Bricelj & Shumway 1991). Therefore, it is suggested that if *N. nodosus* is cultured in open areas of southern Brazil, growth will be enhanced if the culture nets are suspended in waters above the influence of SACW. Conversely, other species, such as the Pacific oyster *Crassostrea gigas* and the mussel *Perna perna*, which are currently cultured in embayments in southern Brazil, may grow well if cultured under SACW influence. Unlike scallops, intertidal bivalves are usually more tolerant of temperature variability and high PIM levels (Bayne 1976, Newell & Langdon 1996, Shumway 1996). The influence of environmental instability on bivalve growth associated with SACW intrusions requires further examination before aquaculture expands further to deeper waters off southern Brazil.

In the current study, postlarval scallops cultured at a higher stocking density (340/bag) displayed a slight but statistically significant reduction in growth compared with those grown at lower density (150/bag) at both depths in experiment 1. Grecian et al. (2000), however, found no difference in growth of *Placopecten magellanicus* at stocking densities of 2600 and 5200 spat/bag, but the surface area of the spat bags and the mesh opening were larger than those used in the current study, so a direct comparison is difficult. A negative relationship between growth and stocking

density inside culture nets has been described for other scallops (Duggan, 1973, Ventilla 1982, Parsons & Dadswell 1992, Côté et al. 1993, Côté et al. 1994, Maeda-Martinez et al. 1997, Román et al. 1999). Parsons & Dadswell (1992) postulated that at higher stocking densities, intraspecific competition for food reduces its availability per individual, limiting growth. Higher densities also lead to frequent contact among organisms, which may induce irritation, retraction of the mantle, or valve closure, resulting in reduced feeding rates, and may also cause breakage of shell margins (Côté et al. 1993). Within the range of densities tested, the reduction in growth at higher density was slight, so it would be more cost-effective to grow postlarval *N. nodosus* at the higher density. This strategy, however, is only appropriate if the spat bags are retrieved after an immersion period of about 26–30 days, as in the current study, when scallops at 4 m in experiment 1 reached a mean size (>5 mm) at which they could be transferred to pearl nets. On the other hand, if the spat bags are to be immersed for longer periods before spat are detached and transferred to pearl nets, it is likely that the density influence on growth will increase as scallops attain larger sizes.

A comparison of daily growth rates can be made between the high density treatment in experiment 1 (350 scallops/bag; SE = 54.7) and experiment 2 at both depths (401 scallops/bag; SE = 95.9), in which stocking densities were equivalent. Though the lowest DGR was recorded in experiment 1 at 12 m (0.110 mm day⁻¹), and the highest at 4 m (0.179 mm day⁻¹), DGR in experiment 2 was not significantly different between depths (0.144 and 0.137 mm day⁻¹ at 4 and 12 m, respectively). Lower growth rates at 12 m in experiment 1 can be explained by differences in environmental factors between depths, as previously noted. Differences in DGR between 4 m in experiment 1 and both depths in experiment 2 can be explained by seasonal differences in temperature, because chlorophyll-*a*, seston, salinity, and oxygen were similar at 4 m in experiment 1 and at both depths in experiment 2 and therefore cannot account for observed differences in growth. Temperature, on the other hand, was significantly lower in experiment 2 (mean = 20.4°C and 20.1°C at 4 and 12 m, respectively) than in experiment 1 at 4 m (mean = 25.6°C). This difference probably accounted for differences in growth between seasons, in accordance with the results of Rupp (2003), which showed that seasonal variation in temperature, rather than food availability, explained differences in DGR of postlarval *N. nodosus*.

Percentage Retrievals

Percentage retrievals of postlarval *N. nodosus* were similar between depths and experiments and ranged from 69.3% to 81.3%. These values are higher than those reported in Heasman et al. (2002) (25.4%) for *Pecten fumatus*, in which postlarval scallops at a shell height of 0.5 mm were deployed in the sea-based nursery attached to monofilament collectors, as in the current study. Stocking density, as well as the variability in environmental factors recorded in the current study, had a negligible effect on retrieval of scallops, even when they experienced sudden temperature changes at 12 m in experiment 1. Dickie & Medcof (1963) reported mass mortalities of adult scallops *Placopecten magellanicus* in Atlantic Canada due to a sudden change in the position of the thermocline. In southern Brazil, *N. nodosus* cultured at 12 m can experience sudden temperature shocks on a semi-diurnal basis in magnitudes similar to the seasonal temperature variation, but such variation did not cause spat mortality. Temperatures in the current study did not reach the lethal levels determined by Rupp & Parsons (in press).

Environmental Variables

During the first 2–3 wk of experiment 1 (March), scallops at 12 m were influenced by an oscillating water mass with thermal characteristics different from the upper layer, with the result that temperature variability at 12 m was greater than at 4 m. A colder bottom water mass frequently influenced the 12 m layer but only occasionally reached 4 m. These differences in water masses between depths resulted in a temperature range of 10.4°C between 4 m and 12 m in less than 24 h, resulting in a semi-diurnal variability in temperature equivalent to that observed on a seasonal time scale (Rupp 2003). Such cold water intrusions disappeared toward the end of experiment 1 (April) and were not observed in experiment 2 (June–July). Furthermore, maximum values of chlorophyll-*a* were recorded in March, when concentrations at 12 m were higher than at 4 m. Such spatial variation in temperature and chlorophyll-*a* is in agreement with the patterns reported in other studies for the inner continental shelf of southern Brazil when SACW is present (Brandini 1990, Borzone et al. 1999). There is strong evidence that scallops held at 12 m in experiment 1 were under the influence of an oscillating water mass that can be identified as predominantly SACW. SACW has been characterized as a cold water mass (10–20°C) affecting lower layers of the continental shelf (Matsuura 1986, Castro & Miranda 1998) with relatively higher concentrations of nutrients and chlorophyll-*a*, lying underneath warmer Coastal Waters (CW), which are relatively oligotrophic (chlorophyll-*a* < 0.4 µg L⁻¹) off Santa Catarina State during summer (Brandini 1990). Borzone et al. (1999) indicated that from December to April (summer–early autumn), the water column on the inner continental shelf displayed a marked thermocline, with near-surface temperatures reaching 27°C, whereas below the thermocline, the temperature was 16°C and a chlorophyll-*a* maximum of ca. 3 µg L⁻¹ was recorded. This gradient was attributed to SACW intrusions. Conversely, from May to September (late autumn–winter–early spring), the temperature gradient disappeared with vertical mixing of the water column (Borzone et al. 1999). During this period, SACW retreats toward the shelf break (Castro & Miranda 1998), and the inner continental shelf becomes influenced by cold (<20°C), low salinity water (29–35‰) of sub-Antarctic origin (SAW), leading to a vertically mixed water column (Sunyé & Servain 1998). These observations are consistent with the recorded differences in temperature between experiments 1 and 2 in the current study. The salinity reported for SACW is around 35‰ (Matsuura 1986, Castro & Miranda 1998), and salinity at 12 m remained close to 33‰ throughout the current study. According to Castro & Miranda (1998), mixing of SACW and CW occurs across the continental shelf during summer. The water resulting from this interaction, retaining the major characteristics of SACW (relatively low temperature and high chlorophyll-*a* but with slightly lower salinity), is probably the water mass that repeatedly affected the culture site at 12 m during summer, causing strong thermal instability. Moreover, during summer, when the upper layers of the inner continental shelf are dominated by warm CW, the only reports of waters colder than 20°C in the South Brazil Bight are for areas affected by SACW (Castro & Miranda, 1998), providing further evidence that the cold waters affecting lower layers of the coastal aquaculture site originated from SACW.

The oscillation of the cold water mass at the study site during summer may have been a result of tidal influence or wind stress. Penetration of SACW onto the continental shelf has been related to situations in which prevailing winds blow offshore, generating a

cross-shelf circulation toward shore in the deeper layers (Castro & Miranda, 1998). However, the short-term variability in the position of SACW has not been investigated in detail. The factors influencing hydrographic processes in shallow coastal areas are complex and vary greatly on short spatial and temporal scales (Mann & Lazier, 1991). The study region is characterized by a microtidal regimen, which may have contributed to the oscillation of the cold water mass, but the small tidal amplitude may not have been sufficient to destabilize the thermocline and generate vertical mixing throughout the water column. The spatial and temporal dynamics of SACW along the southeastern coast of Brazil are still poorly understood, and the implications of SACW intrusions on aquaculture require further study.

Another important environmental factor that markedly differed between 4 and 12 m in experiment 1, but not in experiment 2, was PIM, which was higher at 12 m. No previous study carried out in southern Brazil has investigated the organic and inorganic contents of the seston associated with different water masses. The higher percent PIM near the bottom recorded in experiment 1, but not in experiment 2, is possibly associated with SACW, suggesting that during the process of SACW intrusion into the lower layer across the continental shelf, a frictional shear on the substrate resuspends bottom sediments, resulting in the higher PIM in the SACW. According to Borzone et al. (1999), the bottom sediments of the inner continental shelf of the SBB are formed by quartz sand with a silt-clay fraction that increases with depth. These fine sediments could be resuspended in the water column, increasing PIM levels of SACW. A further indication of the deeper origin of the water mass affecting the bottom layers is dissolved oxygen, which was lower at 12 m on two occasions, once falling below 50%. According to Longhurst & Pauly (1987), waters originating from deeper layers of the ocean often contain less oxygen than surface waters.

In conclusion, the influence of environmental factors associated with depth at an aquaculture site in southern Brazil varied seasonally, depending on the subsurface intrusion of SACW. The characteristics of this water mass were less favorable for postlarval scallop growth than those of near-surface waters. During summer–early autumn, lower temperature and sudden temperature changes, together with a higher inorganic content of the seston associated with SACW, resulted in slower growth of postlarval *N. nodosus* at 12 m than at 4 m. Furthermore, on several occasions, the vertical thermal gradient from 4 to 12 m (10°C) was similar to the seasonal temperature variation. Conversely, during late autumn–early winter, the water column was vertically mixed, and scallop growth was similar at both depths. Higher temperatures explained higher growth rates near the surface during summer–early autumn compared with lower growth in late autumn–early winter at both depths. Furthermore, growth of postlarval scallops cultured in spat bags showed an inverse relationship with stocking density, but the differences were small and it would be more cost-effective to grow postlarval scallops at the higher density tested. It is suggested that during summer–early autumn, if the site is subject to SACW intrusions, suspended nursery culture of scallops should be undertaken above the thermocline to maximize growth.

ACKNOWLEDGMENTS

This research was supported by a CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) Ph.D. scholarship to G.S.R. and by the Brazilian Mariculture Linkage Program (BMLP), Canada. The Laboratory for the Culture of Marine Mol-

luses (LCMM). Federal University of Santa Catarina, provided experimental facilities and technical assistance. We particularly thank Cheryl Brooking and Marcos Albuquerque for help with the

experiments and Drs. Pat Dabinett, Sandra E. Shumway, David Innes, and Don Deibel for valuable comments on an earlier version of the manuscript.

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COMPARISON OF BIOCHEMICAL COMPOSITION AND MUSCLE HYPERTROPHY ASSOCIATED WITH THE REPRODUCTIVE CYCLE OF DIPLOID AND TRIPLOID SCALLOPS, *ARGOPECTEN VENTRICOSUS*

E. PALACIOS,* I. S. RACOTTA, A. M. IBARRA, J. L. RAMÍREZ, A. MILLÁN AND S. AVILA
Programa de Acuicultura, Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Mar Bermejo 195, Col. Playa Palo de Santa Rita, La Paz, B.C.S. 23090, México

ABSTRACT Triploid organisms have a limited capacity to develop gonads, and thus are considered sterile or partially sterile. The objective of this study is to compare triploid and diploid organisms of the same age, and grown under similar conditions during a 1-year period, to determine to what extent sterility affects size and number of adductor muscle cells, and accumulation of biochemical reserves at first maturation. Adult catarina scallops (*Argopecten ventricosus*) were matured, and eggs were treated with cytochalasin-B to induce triploidy. Untreated diploid controls and triploids were grown in Bahía Magdalena, Mexico in Nestier trays at 3 m depth from April 2000 to March 2001. Scallops were sampled monthly from June 2000. The gonadosomatic index (GSI) was significantly larger in diploid organisms, whereas the muscle index was larger in triploid organisms. A small proportion (40%) of diploid scallops exhibited reproductive activity during the first year. In spite of high GSI, the gonads of triploid scallops were mostly immature, except for some individuals that formed mature oocytes by March 2001. Triploids had 123% higher adductor muscle weight than diploid scallops at the end of the sampling period, and most of this increased gain was a result of adductor muscle cell size (hypertrophy), and not cell number (hyperplasia). Total lipids and proteins concentrations were significantly larger in the gonads of diploid scallops. Muscle carbohydrates were significantly larger in triploid scallops, which suggests decreased transference of carbohydrates from muscle to gonad in triploid sterile organisms.

KEY WORDS: energy-of-allocation, gonad, maturation, muscle, oocytes, Pectinidae, reproduction, *Argopecten*

INTRODUCTION

During the reproductive period of pectinids, there is an important accumulation of lipids and proteins in the gonads (Barber & Blake 1981, Barber & Blake 1985, Martínez 1991, Pazos et al. 1996), mostly provided by food or mobilization of endogenous reserves when food is not readily available (Barber et al. 1991). The adductor muscle of pectinids is the site of one of the largest reserves of glycogen and protein mobilized during maturation (Barber & Blake 1981, Barber & Blake 1985, Epp et al. 1988). When reproductive activity begins, there is a large transfer of biochemical components to the gonad, and the weight of the muscle decreases. Also, water content in the adductor muscle is modified, and this can affect its taste and texture and thus its market price (Allen & Downing 1991). Triploid organisms have limited capacity to mature, and in most species where triploidy is attained, individuals do not produce gametes or viable larvae, and are considered sterile. An advantage of sterile organisms is that they transfer less biochemical energy to the gonad, so other tissues can gain more weight over time. For this reason, triploidy has been induced in pectinids (Tabarini 1984, Beaumont 1986, Komaru & Wada 1989, Ruiz-Verdugo et al. 2000, Yang et al. 2000, Maldonado-Amparo et al. 2004) and other mollusks of commercial interest (for review, see Beaumont & Fairbrothers 1991). The adductor muscle weight of triploid catarina scallops can be 182% that of diploids after 280 days of grow-out (Ruiz-Verdugo et al. 2000). In catarina scallop, reproduction has a significant impact on meat (muscle), because commercial scallops are harvested at 1 year (6-cm length), and first maturation age can be as early as 4 months or 2-cm length (Cruz et al. 2000). Although triploid muscle weight gain is assumed to result from less transfer of biochemical energy from the muscle to the gonads (Ruiz-Verdugo et al. 2001b), no studies have analyzed the effect of increased muscle energy re-

serves on muscle fiber growth (hypertrophy) or number (hyperplasia). An increase in muscle fiber numbers or size can have different effects on the texture of the muscle. The objective of this study is to compare triploid and diploid organisms of the same age and grown under the same conditions during a 1-year period, to determine to what extent sterility affects the biochemical reserves accumulated in muscle, and the size of adductor muscle cells.

MATERIAL AND METHODS

Adult catarina scallops (*Argopecten ventricosus*) were matured under laboratory conditions at CIBNOR, as described by Ramírez et al. (1999). For triploid induction, eggs were treated with 0.5 mg/L cytochalasin-B (CB) during 15 min. after 50% of eggs were in the first polar division, as described by Ruiz-Verdugo et al. (2000). Untreated diploid controls and treated organisms (containing triploids and diploids) were transported to Laguna Rancho Bueno (Bahía Magdalena, B.C.S., Mexico) where they were grown separately in Nestier trays at 3 m depth (Ruiz-Verdugo et al. 2000).

A total of 30 scallops (from both CB-untreated and treated groups) were collected at approximately monthly intervals from the grow-out area at Laguna Rancho Bueno from June 2000 to March 2001 and transferred to the laboratory at CIBNOR. Upon arrival, total live animal weights, and shell-free tissue weights (biomass) of all samples were recorded. Adductor muscle and gonad weight were recorded from July 2000 to the end of the study in March 2001. Organ indices for the two tissues were calculated as the proportion of organ wet weight to total tissue weight minus gonad weight, to eliminate any effect of on-going maturation.

$$\text{GSI (\%)} = (\text{gonad wet weight (g)} \times 100) / (\text{total wet biomass (g)} - \text{gonad wet weight (g)})$$

$$\text{MSI (\%)} = (\text{muscle wet weight (g)} \times 100) / (\text{total wet biomass [g]} - \text{gonad wet weight [g]})$$

Triploidy condition was verified individually in samples of mantle tissue using flow cytometry (Maldonado-Amparo et al. 2004). Un-

*Corresponding E-mail: epalacio@cibnor.mx

treated control organisms and CB-treated diploid scallops were grouped together for statistical analyses. True triploids ranged from 60% to 93.3% in the CB-treated group along each sampling period (Fig. 1), which is similar to the induced triploids of catarina scallop reported by Ruiz-Verdugo et al. (2001a).

Gonad development was evaluated macroscopically during sampling based on stages described by Sastry (1968)—immaturity, partial maturity, maturity, and spent—but including also a “non-active” stage. In addition, a portion of the gonad was fixed in formaldehyde, embedded in paraffin-paraplast mixture, sectioned (6–8 μm), and stained with Harris hematoxylin-eosin (Humanson 1972). Maturation stages of 10 to 15 scallops per sampling were assessed using the classification for triploid and diploid organisms of this species proposed by Ruiz-Verdugo et al. (2000a), and Maldonado-Amparo and Ibarra (2002a).

A portion of the adductor muscle was fixed in 10% formaldehyde, embedded in paraffin-paraplast mixture, sectioned transversely (4–6 μm), and stained with Harris hematoxylin-eosin (Humanson 1972). The number of fibers in a fixed area of striated muscle in diploid and triploid scallops was counted. The diameter of individual striated muscle fibers ($n = 30$ fibers) was assessed using an image analyzer (Image-Pro), and photographed using a microscope ($\times 40$). It was observed that the surrounding connective tissue allowed for an increase in muscle fiber size and it was assumed that this increase was not affecting the number of cells/area.

Gonad and muscle samples were homogenized with a mechanical homogenizer in 1.5 mL of cold, saline solution (NaCl, 35 g/L) to obtain a crude extract, from which analyses for total protein, carbohydrates, and lipids, were done as described in previous works (Ruiz-Verdugo et al. 2001b, Racotta et al. 2003). Lipids were analyzed by the sulpho-phosphovanillin method after mixing 100 μL of the crude extract with 1-mL sulfuric acid and heating

the mixture to 90 °C (Barnes & Blackstock 1973). The amounts of gonad in July and August 2000 samples were not sufficient for analysis. Carbohydrates were analyzed after precipitating proteins from the crude extract with 20% trichloroacetic acid (1:2) and centrifuging at $3000 \times g$, 5 °C for 10 min and mixing the supernatant with four parts of anthrone solution (0.1% dissolved in 76% sulfuric acid), incubated 3 min at 90 °C and cooled to 4 °C to stop further reaction (Van Handel 1965). Soluble proteins were determined in diluted crude extract (1:5 with 0.1N NaOH) (Bradford 1976). The energy conversion factors used were 17.2 kJ/g for carbohydrates, 17.9 kJ/g for proteins, and 33.0 kJ/g for lipids, as described in Heras et al. (1998).

Data are reported as mean \pm standard error (S). Two-way analyses of variance (ANOVA) followed by a Tukey test for unequal n post-hoc mean comparisons (Statistica Version 5.0) were used to assess significant differences among months of sampling and ploidy groups. The level of significance was set at $P < 0.05$. Organ indices were arcsine transformed for the analyses (Sokal & Rohlf 1981), but untransformed data are presented. When there was a significant interaction, a Tukey post hoc to evaluate for differences between diploid and triploid means was performed.

RESULTS

Scallop growth in terms of live weight and tissue biomass are shown in Figure 1. Total or live weight increased continuously from 0.6 g in June 2000, to 33.3 g for diploids and 62.5 g for triploids in March 2001, but significant differences were seen from November 2000 on. Biomass represented 36% of total weight in diploids and 45% in triploids by the end of the sampling period. In March 2001, the biomass of triploids was 88% higher than that of diploids, but biomass differences between diploids and triploids were significant by November 2000.

Gonad weights began to increase earlier in diploid than in triploid scallops, but triploid gonads reached larger weights by March 2001, 2.1 g or 61% higher than the 1.3 g of diploids at that same time (Table 1). Triploid scallops had generally lower gonadosomatic indices (GSI), and higher gonad water content than diploids ($P < 0.01$). Gonad carbohydrates decreased in the triploid group throughout the sampling period, and although the two were not significantly different, the interaction was. In general, there was a greater variation in gonad carbohydrate for triploids than for diploids, with the highest and lowest values both for triploid scallops in October 2000 and February 2001, respectively, which were significantly different to those in the diploid group. Protein and lipid concentrations in the gonads of diploid and triploid scallops were similar from September 2000 to January 2001, but by February 2001 proteins and lipids in diploid gonads increased significantly, decreasing again in March. In the gonads of triploid scallops no increase in protein and lipid was seen in the same period. Energy reserves were similar in gonads of diploid and triploid scallops from September 2000 to January 2001, but increased significantly in gonads of diploid scallops by February 2001, when energy reserves were the lowest ones for triploid scallops, and remained still higher than in triploids in March 2001.

Adductor muscle weight of triploids was 123% greater than that of diploids by the end of the sampling period (Table 2). The muscle index was larger in triploid organisms, except at the beginning of the sampling period, when it was similar to that of diploid scallops. Water content in diploid and triploid muscle was not significantly different, but showed differences related to sampling time, with the lowest values in January 2001 for both groups.

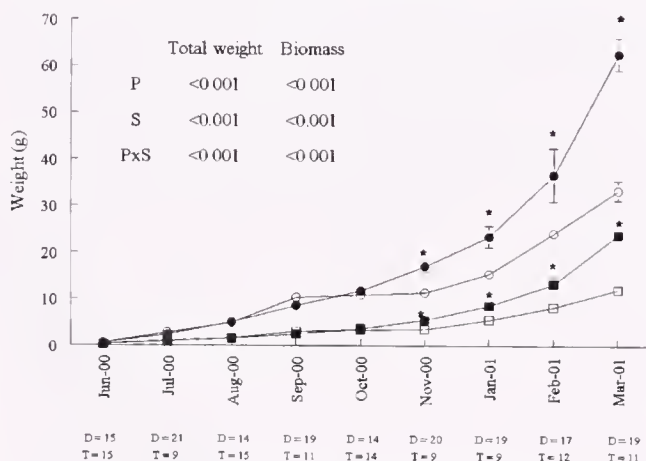


Figure 1. Growth (mean \pm S) of diploid and triploid catarina scallops (*Argopecten ventricosus*). Open circles = diploid total live weight; solid circles = triploid total live weight; open squares = diploid tissue biomass; solid squares = triploid tissue biomass. Data were analyzed with a two-way ANOVA, with ploidy groups as the first main factor ($P = 2$ levels, diploid and triploid scallops), and date of sampling as the second main factor ($S = 9$ levels). The number of diploid (D) and triploid (T) organisms sampled each month is shown at the bottom of the figure. The diploid group includes untreated and CB-treated scallops (see Methods). Significant differences ($P < 0.05$) between diploid and triploid means, as determined by a Tukey post hoc test, are shown with an asterisk.

TABLE 1.

Gonad water content, and biochemical composition (mean \pm S) of diploid (D) and triploid (T) catarina scallops.

		Jul-00	Aug-00	Sep-00	Oct-00	Nov-00	Jan-01	Feb-01	Mar-01	P	S	S \times P
Gonad weight (g)	D	0.07 \pm 0.01a	0.09 \pm 0.01a	0.23 \pm 0.03a	0.28 \pm 0.03ab	0.26 \pm 0.03a	0.31 \pm 0.04ab	0.67 \pm 0.05bc	1.32 \pm 0.15d	<0.01	<0.01	<0.01
	T	0.05 \pm 0.01a	0.07 \pm 0.02a	0.09 \pm 0.02a	0.19 \pm 0.03a	0.27 \pm 0.04ab	0.47 \pm 0.09ab	1.04 \pm 0.23cd	2.08 \pm 0.23e			
GSI (%)	D	8.3 \pm 0.8b	5.6 \pm 0.8bc	8.0 \pm 0.5b	9.0 \pm 0.6ab	7.9 \pm 0.6b	6.0 \pm 0.5b	8.9 \pm 0.5b	12.1 \pm 1.0a	<0.01	<0.01	N.S.
	T	4.7 \pm 0.6bc	4.2 \pm 0.5c	3.7 \pm 0.4c	5.4 \pm 0.4bc	5.0 \pm 0.4bc	5.6 \pm 0.6bc	8.0 \pm 1.0b	9.6 \pm 0.9ab			
Water content (%)	D			81.0 \pm 0.4a	79.9 \pm 0.4ab	78.6 \pm 0.4b	77.7 \pm 0.2b	80.0 \pm 0.2ab	80.3 \pm 0.6ab	<0.01	<0.05	N.S.
	T			81.1 \pm 0.5a	79.7 \pm 0.7ab	80.6 \pm 0.5a	80.1 \pm 1.0ab	80.3 \pm 0.6ab	82.2 \pm 0.7a			
Carbohydrate (mg/g)	D			15.7 \pm 0.9b	11.5 \pm 1.6b	11.8 \pm 0.9b	10.7 \pm 0.5b	10.1 \pm 0.6b	8.2 \pm 1.1b	N.S.	<0.01	<0.01
	T			20.9 \pm 3.9bc	22.4 \pm 5.8c	12.4 \pm 2.5b	8.8 \pm 0.9b	4.5 \pm 0.9a	8.4 \pm 1.1b			
Protein (mg/g)	D			68.7 \pm 2.6ab	72.4 \pm 3.7ab	68.7 \pm 4.1ab	54.5 \pm 3.8a	137 \pm 10.8c	102 \pm 9.6b	<0.01	<0.01	<0.01
	T			70.0 \pm 12.9ab	52.7 \pm 5.3a	59.9 \pm 6.0a	44.7 \pm 6.5a	33.4 \pm 5.0a	67.5 \pm 7.4ab			
Total Lipids (mg/g)	D			8.9 \pm 0.4b	10.3 \pm 0.4b	9.9 \pm 0.5b	8.2 \pm 0.6ab	15.2 \pm 0.7c	10.8 \pm 1.7b	<0.01	N.S.	<0.01
	T			6.3 \pm 0.8ab	7.3 \pm 0.5ab	8.6 \pm 1.8ab	6.1 \pm 0.9ab	3.5 \pm 0.5a	4.8 \pm 0.8a			
Energy (kJ/g)	D			1.79 \pm 0.06bc	1.82 \pm 0.07bc	1.76 \pm 0.10bc	1.43 \pm 0.09ab	3.13 \pm 0.19d	2.32 \pm 0.23c	<0.01	<0.01	<0.01
	T			1.82 \pm 0.28bc	1.63 \pm 0.17b	1.57 \pm 0.27ab	1.15 \pm 0.15ab	0.79 \pm 0.11a	1.51 \pm 0.16ab			

Data were analyzed by a 2-way ANOVA, with ploidy as the first main factor (P, two levels), and sampling date as the second main factor (S, eight levels except July and August, when sample was insufficient for biochemical analyses).

N.S., Not significantly different. When there was a significant interactions, a Tukey for unequal N post hoc test was applied to compare means between groups, and means with different letters are significantly different ($P < 0.05$).

Muscle carbohydrates and lipids varied seasonally in both ploidy groups, but were generally higher in triploid scallops. In contrast, no differences between diploid and triploid scallops in muscle protein and energy reserves were observed, although seasonal variations were observed in both, with the highest values in October 2000.

Gonad development of diploid scallops varied seasonally, with reproductive activity in July to August and in October 2000, although the distribution of gonads into different macroscopic stages showed mature and spent gonads throughout the sampling period (Fig. 2A). In contrast, triploid scallops were mostly inactive or immature, with partially mature gonads in October 2000, and January and March 2001, and mature gonads only in March 2001. However, no spent gonads were found in triploids during the sampling period. Gametogenesis evaluated in diploid and triploid gonads of catarina scallops during 382 days did not reveal maturation in gonads of triploid scallops (Ruiz-Verdugo et al. 2000). The distribution of the macroscopic stages was in accordance with the

maturation stages evaluated by histology (Maldonado-Amparo & Ibarra 2002a).

The adductor muscle cells of triploids were significantly larger than those of diploids on all sampling dates (Fig. 3A). In January 2001, triploid muscle cells were 53% larger than muscle cells of diploid scallops. The largest difference in cell size was observed in February 2001, when the triploid muscle cell size was three times larger than that in diploids. In March 2001, the cells of triploid scallops increased 31% compared with February, and were 79% larger than those of diploid scallops. In contrast, the number of muscle fibers per area (fiber density) was not significantly different between ploidy groups or as a result of sampling time (see Fig. 3B).

DISCUSSION

One advantage of producing triploid mollusks is that individuals grow more (Allen & Downing 1986, Child & Watkins 1994,

TABLE 2.

Muscle water content and biochemical composition (mean \pm S) of diploid (D) and triploid (T) catarina scallops.

		Jul-00	Aug-00	Sep-00	Oct-00	Nov-00	Jan-01	Feb-01	Mar-01	P	S	S \times P
Muscle weight (g)	D	0.29 \pm 0.02a	0.41 \pm 0.04a	0.93 \pm 0.06ab	1.03 \pm 0.07ab	1.01 \pm 0.11ab	1.49 \pm 0.11b	2.22 \pm 0.12c	3.37 \pm 0.22d	<0.01	<0.01	<0.01
	T	0.31 \pm 0.06a	0.47 \pm 0.06a	0.87 \pm 0.08ab	1.28 \pm 0.16b	1.85 \pm 0.22bc	2.68 \pm 0.32cd	3.75 \pm 0.39d	7.53 \pm 0.40e			
Muscle somatic index (%)	D	32.3 \pm 1.5ab	31.0 \pm 1.0b	32.8 \pm 0.9ab	33.2 \pm 1.0ab	30.7 \pm 0.8b	30.0 \pm 1.3b	29.2 \pm 0.5b	31.7 \pm 1.4ab	<0.01	<0.01	N.S.
	T	32.0 \pm 0.8ab	30.7 \pm 1.0b	35.6 \pm 0.7a	36.0 \pm 1.6a	35.2 \pm 0.9a	33.1 \pm 0.7ab	30.6 \pm 0.7b	34.7 \pm 0.6ab			
Water content (%)	D	80.4 \pm 0.5a	80.3 \pm 0.5a	80.1 \pm 0.6a	79.0 \pm 0.2ab	78.5 \pm 0.2ab	76.7 \pm 0.2b	80.0 \pm 0.2a	78.7 \pm 0.4ab	N.S.	<0.01	N.S.
	T	80.8 \pm 0.3a	80.8 \pm 0.7a	80.7 \pm 1.0a	78.6 \pm 0.9ab	77.4 \pm 0.5b	76.3 \pm 0.4b	80.3 \pm 0.6a	78.1 \pm 0.6ab			
Carbohydrate (mg/g)	D	16.0 \pm 0.8b	6.1 \pm 0.6c	21.7 \pm 2.0b	20.3 \pm 2.5b	6.3 \pm 0.9c	1.7 \pm 0.2c	15.9 \pm 1.2b	24.0 \pm 2.4b	<0.05	<0.01	N.S.
	T	19.2 \pm 2.5b	5.5 \pm 1.1c	24.9 \pm 2.5ab	26.9 \pm 3.1a	8.1 \pm 1.1c	3.0 \pm 0.5c	14.8 \pm 1.8bc	28.0 \pm 3.2a			
Protein (mg/g)	D	114 \pm 1.9ab	115 \pm 5.6ab	127 \pm 3.0a	135 \pm 2.6a	120 \pm 3.4ab	133 \pm 5.0a	111 \pm 4.6b	114 \pm 5.1ab	N.S.	<0.01	N.S.
	T	103 \pm 7.9b	118 \pm 2.8ab	126 \pm 5.1a	135 \pm 4.8a	115 \pm 4.1ab	134 \pm 6.5a	115 \pm 5.1ab	125 \pm 7.9ab			
Total Lipids (mg/g)	D	0.26 \pm 0.02ab	0.35 \pm 0.05ab	0.28 \pm 0.06ab	0.35 \pm 0.13ab	0.53 \pm 0.04a	0.25 \pm 0.04b	0.09 \pm 0.02b	0.10 \pm 0.02b	<0.05	<0.01	N.S.
	T	0.34 \pm 0.05ab	0.31 \pm 0.02ab	0.18 \pm 0.03b	0.50 \pm 0.15ab	0.75 \pm 0.20a	0.56 \pm 0.16a	0.17 \pm 0.04b	0.09 \pm 0.01b			
Energy (kJ/g)	D	2.32 \pm 0.04b	2.17 \pm 0.10b	2.67 \pm 0.05a	2.78 \pm 0.06a	2.27 \pm 0.06b	2.42 \pm 0.09b	2.26 \pm 0.08b	2.46 \pm 0.10b	N.S.	<0.01	N.S.
	T	2.19 \pm 0.14b	2.21 \pm 0.07b	2.67 \pm 0.08a	2.90 \pm 0.09a	2.22 \pm 0.08b	2.47 \pm 0.12ab	2.31 \pm 0.09b	2.72 \pm 0.15a			

Data were analyzed by a 2-way ANOVA, with ploidy as the first main factor (P, two levels) and sampling date as the second main factor (S, eight levels).

N.S., Not significantly different. When there was a significant interactions, a Tukey for unequal N post hoc test was applied to compare means between groups, and means with different letters are significantly different.

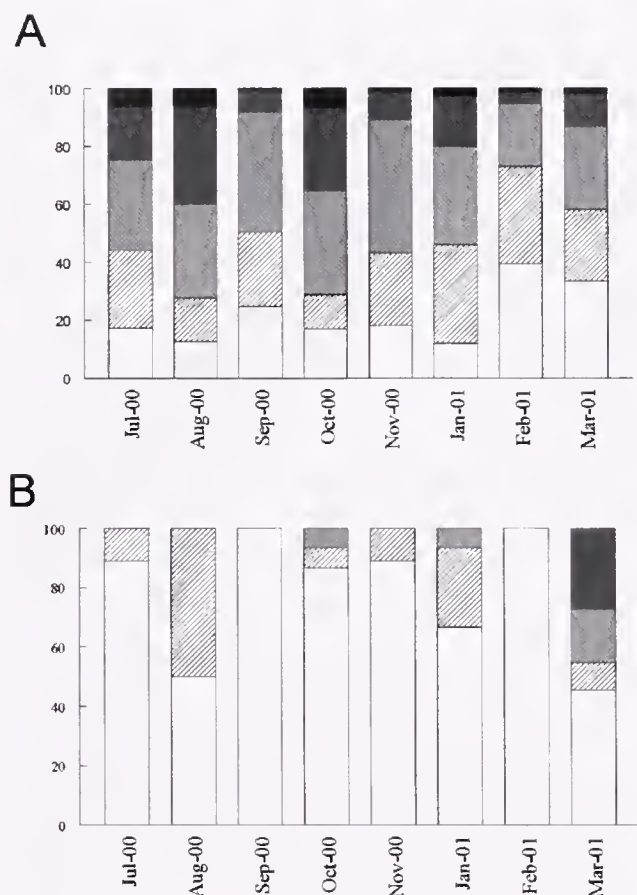


Figure 2. Frequency of diploid (A) and triploid (B) scallops classified into five macroscopic gonadic stages during the sampling period. I = Inactive (open); II = Immature gonad (single line); III = Partial maturation (double line); IV = Mature gonad (crossed lines or hatched); V = spent gonad (black).

Hawkins et al. 1994, Hand et al. 1998, 1999, Kesarcodi-Watson et al. 2001). Several hypotheses have been proposed to explain the higher growth rate of triploids over diploids (Garnier-Géré et al. 2002). A genetic hypothesis is based on the expected higher heterozygosity of triploids (Guo & Allen 1994, Hawkins et al. 2000). Triploids may perform better because of the potential for faster transcription due to the three copies of the same gene (Magoulas et al. 2000). A physiologic hypothesis is based on the sterility of triploids, which would divert more metabolic flux to growth (Allen & Downing 1986, Hand et al. 1999). Guo and Allen (1994) proposed a third hypothesis (i.e., that greater growth in triploid mollusks results from larger size polyploid cells) based on the assumption that a larger nucleus requires a larger cytoplasm so that nutrients and organelles are adequately proportioned during cell division and growth.

Guo and Allen (1994) hypothesized that this resulted in individuals with polyploid gigantism, caused by increased cell volume and a lack of cell number compensation. They stated that the polyploidy gigantism hypothesis needs to be tested directly by studies on cell size, cell number, and organ size in diploids and triploids. Differences in cell size between triploid and diploid mollusks have been reported for eggs (Guo & Allen 1994, Eversole et al. 1996, Ruiz-Verdugo et al. 2001a), sperm (Maldonado-Amparo & Ibarra, 2002b), adductor muscle diameter (Gardner et al. 1996),

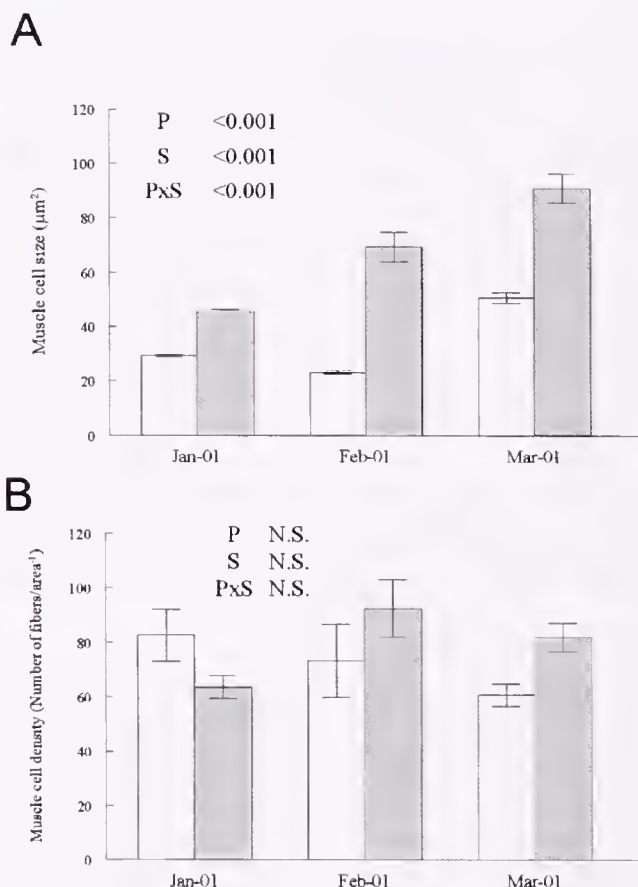


Figure 3. Cell size (A) and cell density (B) of adductor muscle fibers in diploid (white bars) and triploid (single line bars) scallops, analyzed with a two-way ANOVA, considering Ploidy (P = 2 levels: diploid and triploid scallops), and Sampling period (S = 3 levels: January, February, and March of 2001). Anova results are shown in the graph. Means sharing the same letter are not significantly different ($P > 0.05$).

gill tissue cells and hemolymph cell nuclei (Child & Watkins 1994). However, polyploid gigantism might be more apparent in some tissues than in others. For example, in bivalves, the adductor muscle seems particularly susceptible to polyploid gigantism (Guo & Allen 1994). In the present study, we observed that triploid catarina scallops had significantly larger muscle cells diameter (hypertrophy) than their diploid counterparts grown in similar conditions. However, the number of fibers (hyperplasia) in the adductor muscle of triploids was not significantly different from that of diploids, at least during the period tested. Thus, if the number of muscle cells did not decrease to compensate for hypertrophy, a lack of regulation in number of muscle cells is consistent with the polyploid gigantism hypothesis proposed by Guo and Allen (1994).

However, it is difficult to separate hypertrophy caused by polyploidism, and that caused by an increase in energy accumulated in storage tissues per se. This is because, although in most tissues bigger cells might compensate for fewer cells, storage tissues can follow a different pattern. As an example, the adipose tissue in mammals presents hypertrophy as a result of acylglycerides storage (Palacios et al. 1996). This usually happens in adult animals, and it is not accompanied by a decrease in cell number, although there can be an increase in the number of fat cells (hyperplasia). In this work, muscle carbohydrates in triploids increased 5-fold from

January to February, and 2-fold from February to March 2001 (Table 2), corresponding to similar increases in muscle cell size during the same times (see Fig. 3A). In pectinids, where muscle cells are implicated in the storage and mobilization of nutrients to meet reproductive requirements (Mathieu & Lubet 1993), the observed glycogen storage in triploid adductor muscle could be a normal mechanism to accumulate excess energy during periods of high productivity. Thus, we should expect muscle hypertrophy when the conditions are adequate even in diploids, as was the case for diploids sampled at the end of the experiment and compared with those sampled in January 2001 (see Fig. 3A). Therefore, an increase in cell size can be compatible with either larger polyploid cell volume or changes in allocation of energy reserves from muscle to gonad.

The energy allocation hypothesis is based on the sterility or partial sterility of triploid mollusks, and is characterized by triploids growing larger because of energy diverted from reproduction to growth (Stanley et al. 1984, Allen & Downing 1986, Akashige 1990, Barber & Mann 1991, Shpigal et al. 1992, Hawkins et al. 1994, Hand et al. 1998). Differences in growth are not usually detected until the organisms reach first sexual maturity, or until after first spawn (Stanley et al. 1984, Tabarini 1984, Barber & Mann, 1991, Beaumont & Fairbrothers 1991, Hand et al. 1998, Ruiz-Verdugo et al. 2001b). The losses attributable to spawning in a 40-mm shell length hard clam had been calculated by Ansell and Lander (1967) at 20% to 25% of the total energy used for growth. Eversole et al. (1996) concluded that this amount of energy diverted into growth rather than reproduction may account for the difference in size between diploids and triploids. In accordance, we observed higher energy values in the gonads of diploid scallops by the end of the sampling period (see Table 1) than can be interpreted as energy loss for growth. Guo and Allen (1994) concluded that unless diploids spawned thus decreasing their total weight, and triploids did not, the energy reallocation hypothesis could not explain the larger total weight in triploids before sexual maturation. However, in pectinids the adductor muscle is the most important storage tissue with the digestive gland secondary, shown by the decrease in weights during the reproductive period (Barber & Blake 1981, 1985, Epp et al. 1988, Couturier & Newkirk 1991, Martínez 1991, Pazos et al. 1997), and the effects of maturation on weight and condition index of the adductor muscle are significant. For example, Barber and Blake (1981) have shown that in *A.*

irradians adductor muscle dry weight decreased by two-thirds during maturation. Muscle weight decline has been associated mostly with decreases in muscle glycogen, and on increase in gonad lipids (Comely 1974, Taylor & Venn 1979). A diploid scallop transforming muscle glycogen to lipids theoretically could lose wet weight during maturation without releasing eggs. This is because glycogen can be converted to lipids through lipogenesis in mollusks (Gabbot 1975), but glycogen is stored in a highly hydrated form; for each gram of stored carbohydrates, there are 4 to 5 g of stored water (Randall et al. 1998). In contrast, lipids are accumulated in tissues with very little water. Thus, to produce 1 g of lipid, an organism must use a much larger weight of glycogen. We observed that carbohydrate concentrations were lower in the muscle of diploid organisms in October 2000 and March 2001, whereas lipid concentrations in the gonads increased by the end of the sampling period, as expected for organisms going through gametogenesis-vitellogenesis and previously observed for the same species (Ruiz-Verdugo et al. 2001b). With no difference in carbohydrates and less total proteins and lipids in the gonads of triploid organisms, we might have expected a smaller gonad, but water content in triploid gonads was higher than in diploids, thus contributing to higher total gonad weight. In muscle of triploid scallops, we expect greater muscle weight, with no differences in water and protein and with more carbohydrates and lipids. Thus, greater total and muscle weights in triploid scallops theoretically could be attained without spawning.

In conclusion, triploid sterility increases adductor muscle weight in catarina scallops, through an increase in carbohydrate storage that affects the size of adductor muscle fibers (hypertrophy), which is a result of a lower energy allocation to gonads. Thus, the increase in muscle size in the gigantism hypothesis may be a direct consequence of the energy allocation hypothesis.

ACKNOWLEDGMENTS

The authors thank Rosalio Maldonado, Juan H. Macliz, and Gabriel González for support in the sample processing; Carmen Rodríguez-Jaramillo and Teresa Arteché for histologic processing of gonads and muscles; and English editing staff at CIBNOR. The authors also thank two anonymous reviewers for critical comments. This research was supported by SIMAC project BCS7001 and CONACyT project 28256B.

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AFLP LINKAGE MAP OF AN INTRASPECIFIC CROSS IN *CHLAMYS FARRERI*

SHI WANG, ZHENMIN BAO,* JIE PAN, LINGLING ZHANG, BING YAO, AIBIN ZHAN, KE BI AND QUANQI ZHANG

Laboratory of Marine Genetics and Breeding (MGB), Division of Life Science and Technology, Ocean University of China, Qingdao 266003, People's Republic of China

ABSTRACT Zhikong scallop, *Chlamys farreri*, is the most important species for sea-scallop breeding programs in China. The linkage maps of *C. farreri* were constructed with AFLP markers generated from 21 primer combinations. The polymorphism level of F1 progeny is 62.94%. Of the 783 polymorphic markers detected, 317 segregated in 1:1 ratios and 190 in 3:1 ratios, whereas the other 276 showed segregation distortion ($\alpha = 0.05$). Linkage analysis used the program MAPMAKER/EXP at LOD = 2.5 and $\theta = 0.30$. The male map containing 94 markers in 19 linkage groups, was 1511.4 cM in total length and 66.56% in genome coverage. The female map containing 97 markers in 20 linkage groups, was 1610.2 cM in total length and 66.05% in genome coverage. The distribution of AFLP markers is relatively even in chromosomes of male and female map, because only few of clusters were observed.

KEY WORDS: *Chlamys farreri*, genetic linkage map, AFLP, intraspecific cross, segregation distortion

INTRODUCTION

Genetic linkage maps have become powerful research tools in genetic studies of many species (Saliba-Colombani et al. 2000, Wu et al. 2000, Tan et al. 2001). A saturated linkage map can efficiently carry out molecular-based analyses such as molecular marker-assisted selection (Rance et al. 2001, Kelly et al. 2003), quantitative trait loci (QTL) mapping (Linde et al. 2001, Chen et al. 2001, Mignouna et al. 2002), genetic basis of heterosis (Jones et al. 2003, Hua et al. 2003), and comprehensive investigations of genome evolution (Naruse et al. 2000, Doganlar et al. 2002, Slate et al. 2002).

Although there have been many techniques such as RFLP, RAPD, and SSR available for constructing genetic linkage maps, their applications are limited for various reasons. Amplified fragment length polymorphism (AFLP) is a recently developed marker system that combines the advantages of different marker systems and provides a new opportunity for mapping species with large, but less understood genomes. It can detect a large number of genetic loci per reaction and thus can obtain more genetic information than RFLP and RAPD analysis.

AFLP markers have been widely applied to construct the linkage maps in a variety of economically important species, such as rice (Virk et al. 1998, Zhu et al. 1999), rye (Saal & Wricke 2002), soybean (Keim et al. 1997), chicken (Herbergs et al. 1999, Knorr et al. 1999), silkworm (Tan et al. 2001), tea (Hackett et al. 2000), coffee (Ky et al. 2000), fish (Young et al. 1998, Coimbra et al. 2003), oyster (Yu & Guo 2003), and shrimp (Wilson et al. 2002, Li et al. 2003). AFLP markers have also been successfully applied to important genes mapping such as the scab resistance *Vf* gene in apple (Xu & Korban 2000), the *Lr19* gene for resistance to leaf rust in wheat (Prins et al. 2001), the *rhm* gene for resistance to Southern Corn Leaf Blight in maize (Cai et al. 2003), the powdery mildew resistance gene *Rm1* in grapevine (Pauquet et al. 2001), and the fertility restorer gene *rf4* in sorghum (Wen et al. 2002).

Chlamys farreri belongs to Mollusca, Lamellibranchia, Pteriomorphia, Pterioodae, and Pectinidae and distributes mainly in the northern China, the western Korea, and Japan. It is a diploid with 38 chromosomes (Wang et al. 1990) and is one of the most important aquaculture species in China. It is important to understand

its genome organization by means of molecular markers for genetic studies and breeding purposes. However, till now, a linkage map of *C. farreri* has not been reported. The purpose of this study is to build the first AFLP-based linkage map of *C. farreri*.

MATERIALS AND METHODS

Animal Materials

The mapping population of 51 F1 progeny was derived from a pair mating of *C. farreri*. The parents were sampled from a wild population around Changdao Island, Shandong Province, China. The muscle tissues of the parents were scissored off and stored at -20°C . The F1 progenies were sampled at the swimming trochophore larvae stage (about twenty hours after fertilization) and stored in ethanol at 4°C .

DNA Extraction

The parental DNA was extracted from frozen muscle tissues with phenol/chloroform extraction as described by Sambrook et al. (1989). The F1 larvae were individually collected in PCR tube through micro-operation and then lysed in 10- μL sterile water for about 5 hours to serve as the DNA solution.

AFLP Analysis

AFLP analysis was carried out essentially as described by Vos et al. (1995) with some minor modifications, such as reducing the dosage of the reagents, for AFLP procedure in F1 individuals. The DNA samples were digested with *EcoRI* and *MseI*, then ligated to restriction site-specific adaptors. Preamplification was carried out using adaptor-specific primers with no selective base on each primer. The preamplification product was diluted (20-fold for parents and 10-fold for progeny) and then used in selective amplification. The selective amplification used the primers with three selective bases on each primer. Totally, 21 primer combinations were selected for AFLP analysis.

The products of the selective amplification were separated by polyacrylamide gel electrophoresis at 60 W for 1.5 hours and the bands were detected by silver staining. The electrophoretic images were scanned and then saved in a computer for further analysis.

*Corresponding author. E-mail: zmbao@ouc.edu.cn

TABLE 1.
Inheritance of AFLP fragments in the F1 progeny of diploid organisms.

Type	Genetic Model	Expected Ratio	AFLP Phenotypes									
			Parents					Offspring				
1	AA×AA→AA	1:0	—	—	—	—	—	—	—	—	—	—
2	AA×Aa→AA,Aa	1:0	—	—	—	—	—	—	—	—	—	—
3	AA×aa→Aa	1:0	—	—	—	—	—	—	—	—	—	—
4	Aa×AA→AA,Aa	1:0	—	—	—	—	—	—	—	—	—	—
5	Aa×Aa→AA,Aa,aa	3:1	—	—	—	—	—	—	—	—	—	—
6	Aa×aa→Aa,aa	1:1	—	—	—	—	—	—	—	—	—	—
7	aa×AA→Aa	1:0	—	—	—	—	—	—	—	—	—	—
8	aa×Aa→Aa,aa	1:1	—	—	—	—	—	—	—	—	—	—
9	aa×aa→aa	0:1	—	—	—	—	—	—	—	—	—	—

Scoring of Data and Marker Nomenclature

In preliminary study we found that the bands ranged 50–1500 bp, had good reproducibility, and could unambiguously scored, so only these bands were scored in this study. Two persons scored all these bands independently. The consistent data were used for linkage analysis and scored as dominant markers. Bands present were scored as "1" and absent as "0". Unreliable bands were scored as missing "-".

The AFLP marker names were refereed to the primers used: E followed by two numbers refers to the *EcoRI* primer and M followed by two numbers to the *MseI* primer. Bands were numbered serially in descending order of fragment length; thus the last two

numbers of the AFLP marker code refer to the relative position of the band on the gel.

Segregation Analysis

The inheritance patterns of AFLP bands from parents to offspring in a diploid organism are shown in Table 1. Loci segregating in type 1, 3, 7, and 9 are not informative for mapping since all progeny are identical. Type 2 and 4 can only be scored on the basis of band intensities and were thus excluded due to their low reliability. Only type 5, 6, and 8 containing locus segregation information in the F1 progeny can be used for segregation analysis.

For each marker of all 3 types (type 5, type 6, and type 8).

TABLE 2.
AFLP polymorphism of an intraspecific cross in *C. farreri*.

Primer Combination	EcoRI Primer (5'-3')	MseI Primer (5'-3')	Approximate Number of Bands (n)	Polymorphic Bands (n)	Polymorphic Bands (%)
E32M49	E00 ¹ +AAC	M00 ² +CAG	83	58	69.88
E32M54	E00+AAC	M00+CCT	50	31	62.00
E32M55	E00+AAC	M00+CGA	54	34	62.96
E32M58	E00+AAC	M00+CGT	70	47	67.14
E32M61	E00+AAC	M00+CTG	45	33	73.33
E32M48	E00+AAG	M00+CAC	55	44	80.00
E32M58	E00+AAG	M00+CGT	52	27	51.92
E32M61	E00+AAG	M00+CTG	65	18	27.69
E35M55	E00+ACA	M00+CGA	46	21	45.65
E35M61	E00+ACA	M00+CTG	70	25	35.71
E38M48	E00+ACT	M00+CAC	84	60	71.43
E38M58	E00+ACT	M00+CGT	64	45	70.31
E38M61	E00+ACT	M00+CTG	82	51	62.20
E39M48	E00+AGA	M00+CAC	58	44	75.86
E42M54	E00+AGT	M00+CCT	43	31	72.09
E42M58	E00+AGT	M00+CGT	52	41	78.85
E42M61	E00+AGT	M00+CTG	55	29	52.73
E44M61	E00+ATC	M00+CTG	42	25	59.52
E45M55	E00+ATG	M00+CGA	54	36	66.67
E45M58	E00+ATG	M00+CGT	63	41	65.08
E45M61	E00+ATG	M00+CTG	57	42	73.68
Total	—	—	1244	783	—
Average	—	—	59	37	63.08

¹ E00, 5'-GACTGCGTACCAATTC-3'

² M00, 5'-GATGAGTCCTGAGTAA-3'

goodness-of-fit of observed-to-expected allelic ratios was analyzed with χ^2 test ($\alpha = 0.05$). Theoretically all three types should have a binomial distribution (Van der Lee et al. 1997). For type 6 and type 8 markers the probability of band presence in progeny is 0.5 and for type 5 markers, the probability is 0.75. The distribution of the segregation ratios in F1 progeny was used to compare with the expected. The goodness-of-fit of observed-to-expected distribution was also analyzed with χ^2 test ($\alpha = 0.05$).

Linkage Analysis

Type 6 and 8 markers were used to construct separate genetic linkage maps for female and male parents with the software program MAPMAKER/EXP (Whitehead Institute, F2 backcross model). The "error detection" feature was used to recognize the circumstance when an event was more probably the result of error than recombination. This feature avoids map expansion (Cervera et al. 2001). A LOD score of 3.0 and maximum recombination fraction $\theta = 0.30$ were initially set as the linkage threshold for grouping markers. Groups were then analyzed with multipoint mapping functions to define the most likely map orders. Markers that did not significantly depart from Mendelian ratios at $\alpha = 0.05$ level were used in the grouping analysis. Once the framework linkage groups were established, the relatively less stringent criteria (LOD = 2.5 and $\theta = 0.30$) were applied to test whether there were any additional markers or distorted markers that could be mapped to the framework map. Map distance in CentiMorgans was calculated with Kosambi's mapping function. Linkage groups were drawn with the MAPCHART2.1 program.

Map Length and Coverage

Two methods were used to calculate the estimated genome length. First, we calculated the average marker spacing/interval(s) by dividing the total map length by the number of intervals (number of markers minus number of linkage groups). The estimated genome length (G_{el}) was determined by adding 2s to the length of each linkage group to account for chromosome ends (Fishman et al. 2001). Second, an estimated genome length (G_{es}) was calculated by multiplying the length of each linkage group by $(m+1)/(m-1)$, where m is the number of framework markers in each group (Chakravarti et al. 1991). Lastly, the average of the two estimates was used as the estimated genome length (G_e) for *C. farreri*. Observed genome lengths were calculated as the total length considering all markers (G_{oa}). The observed genome coverage C_{oc} was determined by G_{oa}/G_e .

Distribution of AFLP Markers

The distribution of AFLP markers was analyzed from 3 aspects:

First, under the hypothesis that AFLP markers are randomly distributed over a linkage group, a Poisson distribution function was assumed with the average marker number per 10 cM interval (μ) as the expected mean (Saal & Wricke 2002). Any interval on a specific chromosome, where the observed number of AFLP markers exceeded the 99% quantile of the cumulative distribution function, was considered an AFLP cluster.

Second, the AFLP marker distribution was also analyzed by calculating the Pearson correlation coefficient between the number of AFLP markers in the linkage groups and the size of the linkage groups (Yu & Guo 2003). Then t -test was applied to test the significance of correlation coefficient at $\alpha = 0.01$ level.

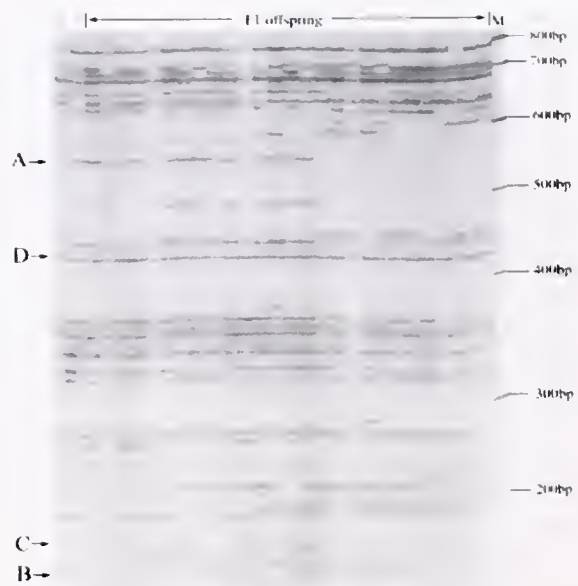


Figure 1. Representative AFLP polymorphism and segregation pattern for 2 parents and 27 progeny with primer combination E33M58. (M is GeneRuler DNA Ladder Mix; A is type 8 marker; B is type 6 marker; C is type 5 marker; D is type 1, 2, 3, 4 or 7 marker)

Lastly, the mapped AFLP markers were classified according to primer combination for analyzing its distribution among the 21 primer combinations used.

RESULTS

Polymorphism Level of AFLP Markers in *C. farreri*

In total, 1244 bands have been detected, of which 783 were polymorphic, that is, 62.94% polymorphic markers. The polymorphism level of each primer combination is listed in Table 2. Depending on the different primer combinations, the number of bands was counted from 42 to 84 in the range of 50 to 1500 bp. Levels of polymorphism individually ranged from 27.69% for E33M61 to 78.85% for E42M58. A typical amplification profile by E33M58 is shown in Figure 1. For all primer combinations, significant cor-

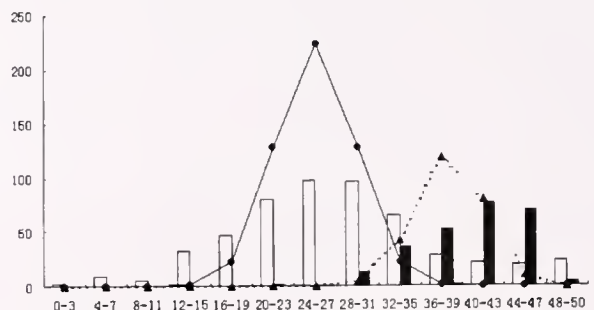


Figure 2. Segregation ratios of AFLP markers in 51 F1 progeny of *C. farreri*. The number of F1 progeny in which an AFLP marker was found was divided into 13 classes (X-axis). The number of AFLP markers (Y-axis) in each class is shown by bars. Open bars represent type 6 and type 8 markers; solid bars represent type 5 markers. The distribution of presence and absence was compared with the expected binomial distribution (curve in solid line, type 6 and type 8 markers; curve in dashed line, type 5 markers).

relation between the total number of bands and the number of polymorphic bands could be observed ($r = 0.68$, $t = 4.04 > t_{0.01}$).

Segregation Analysis

In the 783 polymorphic markers detected, 257 belonged to type 5, of which 190 segregated in 3:1 ratio and 67 showed segregation distortion ($\alpha = 0.05$). The other 526 belonged to type 6 or 8, that is, were segregated in either the male or the female parent. For the male parent, 165 segregated in a 1:1 ratio showing 37.5% segregation distortion, whereas 152 segregated in a 1:1 ratio showing 41.98% segregation distortion for the female parent.

The distribution of the segregation ratios observed in F1 progeny did not resemble the expected distribution ($\alpha = 0.05$) (Fig. 2). The tendency of 1:1 segregation markers' distribution is similar to the expected distribution. However, compared with the expected distribution, the distribution of 1:1 segregation markers showed homozygote excess in the left and heterozygote excess in the right. For the distribution of 3:1 segregation markers, it shows significant allozygote deficiency compared with the expected distribution.

Linkage Map Construction

Two linkage maps were constructed: one for the male parent, the other for the female parent. The male map consisted of 94 markers in 19 linkage groups that covered 1511.4 cM in length, with a maximum interval of 36.9 cM and an average interval of 20.15 cM (Fig. 3; Table 3). The length of the linkage groups ranged from 3.9 to 265.4 cM and the number of markers varied from 2 to 15 per group.

The female map consisted of 97 markers in 20 linkage groups that covered 1610.2 cM in length, with a maximum interval of 36.9 cM and an average interval of 20.91 cM (Fig. 4; Table 4). The length of the linkage groups ranged from 12.0 to 316.0 cM, and the number of markers varied from 2 to 14 per group.

Map Length and Coverage

As shown in Table 5, the genome lengths estimated by two methods were similar, being 2264.21 cM and 2277.1 cM respectively in male, whereas 2428.81 cM and 2446.6 cM respectively in

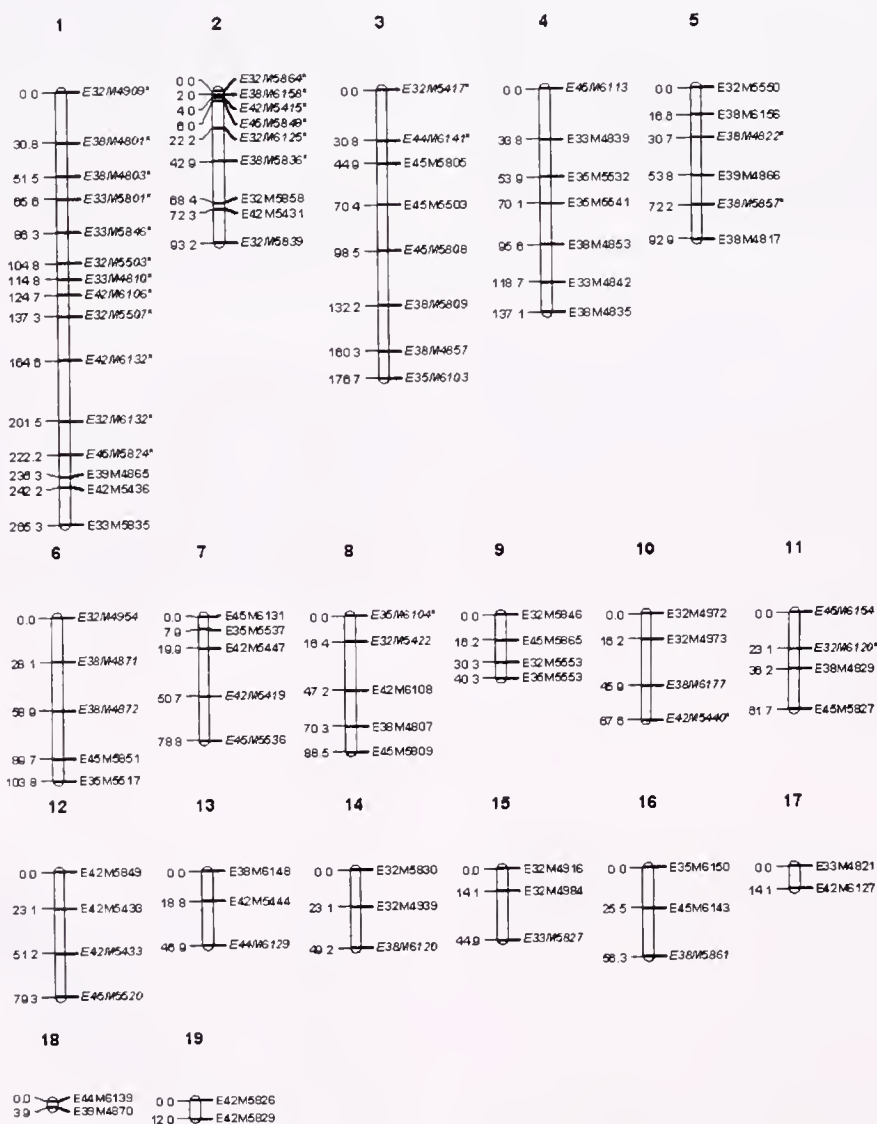


Figure 3. Genetic linkage maps of the male *C. farreri*. (Markers mapped at LOD = 2.5 were shown in italic font and skewed markers were marked by an asterisk)

TABLE 3.

Length, number of markers, average spacing, and largest intervals of linkage groups in the male of *C. farreri*.

Linkage Group	Length (cM)	Number of Markers	Average Interval (cM)	Largest Interval (cM)
1	265.4	15	18.96	36.9
2	93.2	9	11.65	25.5
3	176.8	8	25.26	33.7
4	137.2	7	22.87	30.8
5	93.0	6	18.6	23.1
6	103.9	5	25.98	30.8
7	78.9	5	19.73	30.8
8	86.5	5	21.63	30.8
9	40.3	4	13.43	16.2
10	67.6	4	22.53	29.7
11	61.8	4	20.6	25.5
12	78.3	4	26.1	28.1
13	47.0	3	23.5	28.1
14	49.2	3	24.6	26.1
15	44.9	3	22.45	30.8
16	56.4	3	28.2	30.8
17	14.1	2	14.1	14.1
18	3.9	2	3.9	3.9
19	12.0	2	12.0	12.0
Total	1511.4	94	20.15 ^a	

^a Average of all linked markers.

female. The average of the 2 estimates, 2270.66 cM for male and 2437.7 cM for female, was used as the expected genome length. On the basis of the expected genome lengths, genome coverages of the male and female framework maps were 66.56% and 66.05%, respectively.

Distribution of AFLP Markers

Three clusters were found: two being located on linkage group 2 in male map and one on linkage group 6 in female map. AFLP marker distribution in these regions deviated significantly from the Poisson distribution ($P = 0.01$). The Pearson correlation coefficient analysis (Male: $r = 0.94$, $t = 11.26 > t_{0.01}$, Female: $r = 0.99$, $t = 27.13 > t_{0.01}$) exhibited highly positive correlation between the size of the linkage group and the number of AFLP markers in the linkage group.

The distributions of the AFLP mapping markers among 21 primer combinations were shown in Table 6. The average proportions of markers used for mapping were 35.61% (male) and 37.02% (female), respectively. Most primer combinations generated 5–12 markers that were useful for mapping; but E32M54 and E33M61 generated poorly informative markers for mapping. The numbers of mapped markers among all primer combinations ranged from 2 to 20. Markers generated by any primer combination that were clustered on only one linkage group or in only one region were not found.

DISCUSSION

Polymorphism Level of AFLP Markers in *C. farreri*

The efficiency of constructing a genetic linkage map depends on the heterozygosity level of markers in a species. For *C. farreri*,

its genome should have relatively high heterozygosity because of its mix-mating reproduction system in the nature. In this study, the high polymorphism level (62.94%) as revealed by AFLP markers in the F1 progeny of *C. farreri* reflected high heterozygosity level of the parent genome. The polymorphism level in *C. farreri* based on RAPD markers and isozyme markers is 75.82% and 41.18%, respectively (Song et al. 2002, Li et al. 2001). These results indicate that the heterozygosity level of *C. farreri* is relatively high.

Most of the 21 primer combinations could not only give high polymorphism ratios, but also produce a large number of polymorphic bands. Significant correlation ($r = 0.68$, $t = 4.04 > t_{0.01}$) was found between the total number of bands and the number of polymorphic bands for all primer combinations in the present study, which made it easier to select primer combinations for obtaining highly informative markers.

Segregation Distortion

In this study, segregation distortion ratio is about 35.24% when considering all polymorphic bands in F1 progeny. Segregation distortion ratio is 37.50% (male) and 41.98% (female) when considering 1:1 segregation pattern in either male parent or female parent. Deviations from Mendelian segregation have been reported in constructing genetic linkage maps with molecular markers: 25% of RFLP markers in the soybean (Deshui et al. 1997), and 40% of RAPD markers in the *Medicago* (Jenczewski et al. 1997). Distorted segregation ratios of AFLP markers were also observed: 8.2% in the oyster (Yu & Guo 2003), 14% in the melon (Wang et al. 1997), 32% in the coffee (Ky et al. 2000), 56% in the silkworm (Tan et al. 2001), and 65% in the clubroot (Voorrips et al. 1997). Distorted segregation of molecular markers may result from sampling in finite mapping populations, preferential fertilization, breaking of DNA chains during extraction of DNA samples from tissues, or amplification of a single-sized fragment derived from several different regions (Tan et al. 2001). The above explanations cannot account for the relatively high-distorted segregation in this study. Deviation from Mendelian segregation ratios in bivalves grow more severe as progeny age (Gaffney & Stott 1984), which may result from natural selection against recessive deleterious genes (Launey & Hedgecock 2001). The distribution of 1:1 and 3:1 segregation markers observed in *C. farreri* in this study did not resemble the expected distribution (see Fig. 2) and the fact of significant allozygote deficiency support the hypothesis. Genetic map also provides an efficient approach to investigate the cause of segregation distortion. In this study, the skewed markers assembled together in specific regions of only a few linkage groups, which not only corresponds with the hypothesis but also implies that the specific regions may contain genes influencing the development of the early larvae. Mapping the skewed markers may have little effect on estimating the recombination frequency (Hackett & Broadfoot 2003) but can provide useful information for genetic research and breeding in *C. farreri*, so our final genetic maps include these markers.

Genetic Mapping of AFLPs in *C. farreri*

The haploid genome of *C. farreri* contains 19 chromosomes (Wang et al. 1990). Our male genetic map is composed of 19 linkage groups and female genetic map is composed of 20 linkage groups. Nonequivalence between the number of linkage groups



Figure 4. Genetic linkage maps of the female *C. farreri*. (The symbols are the same as in male map)

and the number of chromosomes has also been reported in other studies (Young et al. 1998, Vivek & Simon 1999). It is difficult to construct maps without missing some chromosomes for species with large number of chromosomes in haploid genomes (Yasukochi 1998), especially when the total number of markers and the coverage are not large enough.

Our AFLP map with an average of 20.15 cM (male) and 20.91 cM (female) per interval was not particularly dense. About 64.39% markers in male and 62.98% in female that show Mendelian segregation were not linked in our maps. Higher proportions of unlinked markers were observed in mapping studies in other species (Grattapaglia & Sederoff 1994). However, these markers may be linked when more markers obtained from other AFLP, RFLP, RAPD, and microsatellite analyses were used to construct denser maps.

Map Length and Coverage

The study provides estimated genetic map length for male and female of *C. farreri* for the first. The male map length is 98.8 cM shorter than the female map length, which may reflect sex-specific recombination rates in *C. farreri*. Similar differences have been noted in aquatic animals including rainbow trout (Sakamoto et al. 2000), zebrafish (Knapik et al. 1998), and Pacific oyster (Yu &

Guo 2003). Several possible explanations, such as time spent in meiotic prophase in one sex, transcriptional activity of certain genes during meiosis in one sex, and presence of sequences that are recognized by sex-specific enzymes (Coimbra et al. 2003), have been applied to explaining the phenomenon. So far, the genetic mechanism of suppression in recombination is not yet clear, and there is no adequate explanation for the difference in recombination rate between the two sexes in *C. farreri*.

The map coverage in the *C. farreri* was 66.56% in male and 66.05% in female. The current map coverage is not high compared with other species. To obtain a saturated genetic map in *C. farreri*, effective resolution is not only to integrate the male and female maps but also to add genetic markers obtained from RFLP, RAPD, EST, and microsatellite analyses as well as isozyme loci into current genetic map.

Distribution of AFLP Markers

The distribution of AFLP markers is relatively even in chromosomes of male and female map, because only few of clusters were observed. The number of AFLP markers in the linkage groups that had high positive correlation with the size of the linkage groups also supports this conclusion. In published literature, AFLP markers distribute randomly in some species (Castiglioni et

TABLE 4.

Length, number of markers, average spacing, and largest intervals of linkage groups in the female of *C. farreri*.

Linkage Group	Length (cM)	Number of Markers	Average Interval (cM)	Largest Interval (cM)
1	316.0	14	24.31	30.8
2	241.5	12	21.95	36.9
3	184.1	10	20.46	25.5
4	133.3	8	19.04	30.8
5	133.2	7	22.2	28.1
6	79.0	6	15.8	25.7
7	73.1	5	18.28	30.8
8	49.2	4	16.4	23.1
9	60.2	4	20.07	24.6
10	51.6	3	25.8	30.8
11	32.7	3	16.35	20.7
12	39.2	3	19.6	20.7
13	44.4	3	22.2	28.1
14	51.7	3	25.85	26.1
15	25.5	2	25.5	25.5
16	12.0	2	12.0	12.0
17	18.4	2	18.4	18.4
18	14.1	2	14.1	14.1
19	25.5	2	25.5	25.5
20	25.5	2	25.5	25.5
Total	1610.2	97	20.91 ^a	

^a The symbols are the same as those in Table 3.

al. 1999, Remington et al. 1999, Cervera et al. 2001), but they form cluster in others (Young et al. 1998, Waldbieser et al. 2001, Sakamoto et al. 2000). Some studies suggest the presence of AT-rich sequence blocks, which are more likely to be recognized by *EcoRI* and *MseI* in connection with suppressed recombination even though such distinct AT-rich regions are not in the centromeric and pericentromeric parts.

Analysis of the distributions of informative markers among primer combinations indicated that neither all primers nor all primer combinations could produce informative polymorphic markers for mapping (see Table 6). This suggests that, similar to the RAPD technique, AFLP analysis of the whole genome such as performed here need to search for primer combinations with highly informative markers.

Applicability of AFLPs in *C. farreri* Genetics and Breeding

Genomic mapping provides a new approach to the identification of genes that control commercially important traits (Dudley

TABLE 5.

Map length and genome coverage for *C. farreri*.

Map Length (cM)	Male	Female
Observed length		
G _{oa}	1511.4	1610.2
Estimate length		
G _{e1}	2277.1	2446.6
G _{e2}	2264.21	2428.81
Average G _c	2270.66	2437.7
Genome coverage (%)		
C _{oa}	66.56	66.05

TABLE 6.

The distribution of the AFLP mapping markers among primer combinations.

Primer Combinations	Male			Female		
	A ^a	B ^b	A/B (%)	A ^a	B ^b	A/B (%)
E32M49	7	22	31.82	5	21	23.81
E32M54	2	10	20.00	0	12	0.00
E32M55	4	12	33.33	7	14	50.00
E32M58	5	17	29.41	3	11	27.27
E32M61	3	12	25.00	3	5	60.00
E33M48	3	16	18.75	9	18	50.00
E33M58	4	6	66.67	4	10	40.00
E33M61	0	2	0.00	2	6	33.33
E35M55	5	8	62.50	0	6	0.00
E35M61	3	10	30.00	4	9	44.44
E38M48	12	23	52.17	8	21	38.10
E38M58	4	12	33.33	6	15	40.00
E38M61	5	21	23.81	2	12	16.67
E39M48	3	11	27.27	4	13	30.77
E42M54	9	11	81.82	5	11	45.45
E42M58	3	11	27.27	7	15	46.67
E42M61	4	13	30.77	1	8	12.50
E44M61	3	11	27.27	1	6	16.67
E45M55	3	9	33.33	7	15	46.67
E45M58	8	17	47.06	10	18	55.56
E45M61	4	10	40.00	9	15	60.00

^a A is the number of mapping markers.

^b B is the number of markers segregating in male or female parent.

1993). AFLPs have the potential to efficiently and rapidly construct high-resolution maps, which will make gene isolation more convenient. The identification and isolation of specific markers closely linked with desirable traits is necessary for marker-assisted breeding. Also, the comparison of genetic maps from different species or genera can provide insights into animal evolution and genome structure (Hohmann et al. 1995).

However, AFLP markers, especially the gel-based, are difficult to transfer among laboratories and populations, which limits the extension of the AFLP maps' application. In some cases, poor transferability can be compensated for by the ease of developing a large set of AFLP markers in any population of interest, thus limiting the need for transfer (Yu & Guo 2003). Microsatellite markers are better markers for linkage mapping than AFLP markers, because of high levels of polymorphism, codominance, and good transferability. At present, our laboratory is developing a set of microsatellite markers in *C. farreri*. In the near future, these microsatellite markers will be contained in the linkage maps, which will enhance the transferability of the current maps.

ACKNOWLEDGMENTS

The authors thank Prof. Guanpin Yang and Dr. Jingjie Hu for reviewing this manuscript. This work is mainly supported by grants 2002AA628110 and 2003AA603022 from Hi-Tech Research and Development Program of China; G1999012009 from "973" Program of China; 30300268 from National Natural Science Foundation of China; 01BS10 from Outstanding Scientists Research Foundation of Shandong Province.

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HYBRIDIZATION EFFECTS OF THREE POPULATIONS OF *CHLAMYS FARRERI* FROM CHINA, KOREA AND JAPAN

LIU XIAOLIN,^{1,3} CHANG YAQING,² XIANG JIANHAI^{1,*}

¹Academy of Sciences, Qingdao 266071, China; ²Key Lab of Marine Ecology in Aquaculture of Ministry of Agriculture, Dalian Fisheries University, Dalian 116023, China; ³College of Animal Technology and University, Northwest Sci.-Tech. University of Agriculture and Forestry, Yangling, Shaanxi 712100, China

ABSTRACT Populations of *Chlamys farreri* are distributed along the coasts of the northern seacoast of China, the Korean Peninsula and the Pacific seacoast, Japan. These populations represent potentially different genetic resources in their production traits important to aquaculture and for their contribution to hybridization breeding programs. To determine their characteristics, crosses between and within three populations of Zhikong scallop, *Chlamys farreri*, were made (Chinese culture population [CC] female × Japanese wild population [JW] male, Japanese wild population [JW] female × Chinese culture population [CC] male, Chinese culture population [CC] female × Korean wild population [KW] male, Japanese wild population [JW] female × Korean wild population [KW] male) and the offspring reared to 24 months of age. Nine consecutive measurements, approximately 60 days apart, were recorded on wet body weight and shell length, width and height. The phenotypic and heterotic response of each sample for growth and survival were compared. The results indicated that there existed positive though varying degrees of heterosis for growth in wet body weight, shell length, shell height, shell width, and survival. Also, there were measurable differences among the reciprocal crosses in their performance traits. The results of this study suggest that hybridization is an important tool to increase the performance and fitness (survival) of cultured scallops.

KEY WORDS: *Chlamys farreri*, hybrid, geographic population, growth, heterosis, hybrid vigor

INTRODUCTION

Chlamys farreri (Jones et Preston), *Chlamys* Roding, *Pectinidae*, *Pterioidea*, *Bivalvia*, *Mollusca* is distributed along the coast of Northern China, the coast of Southern Korea and Northern Korea, the coast of Japan, and the coast of Eastern Russia, it is also widely distributed around the sea area of Shandong province and Liaoning province in China (Pan et al. 2002). The first batch of seedlings were triumphantly cultivated in Dalian, Liaoning province in 1974, and intensively cultured in China where the scallop industry has grown rapidly over the last 20 years. The cultured area has reached 400,000 μ and production yield of 919,591 tons; tip-top yield reached more than one million tons, and *Chlamys farreri* culture is one of the three mainstay mariculture industries in China, which is now one of the top producers of cultured scallop in the world (Xiang 2002). *Chlamys farreri* grow relatively slow (1.5–2 y to market size of 6–8 cm). The primary problem of *Chlamys farreri* culture is cosmically death because of the decline of the idioplasm and disease resistance (Wang et al. 2002). To overcome the obstacle, the new breeding method, especially crossbreeding should be applied.

Technologic developments have made hatchery production of scallop's feasible (Castagna & Duggan 1971, Gruffydd & Beaumont 1972). The scallop species, *Chlamys farreri*, has become an important aquaculture species along the coast of northern China with the first hatchery production of seedling scallops in the 1970s (Wang 1981). However, seedling quality (idioplasm) degeneration and fitness depression have become major technical obstacles to intensive hatchery production of scallops.

Inter-species and intra-specific hybridization has received attention from researchers in aquaculture because it can improve

productivity through hybrid vigor and combine desirable characteristics found in one species with those of another (or in one population with those of another). Experimental attempts to produce inter-species hybrids in the oyster, the scallop, and the hard clam have reported few successes. It has been reported that spawn rate, hatch rate, and the survival of hybrid progeny to post-larval stages are low compared with the intra-specific matings and then some inter-species hybrids are sterile (Hedgecock 1987, Longwell 1987, Menzel 1987, Xiang & Chen 1991, Ouang & Liu 1959, Clark & Talbot 1973, Ke & Tian 2000, Leighton & Lewis 1982, Zhou & Gao 1982).

Intra-specific hybrids can produce heterosis and combine desirable characteristics to improve productivity because different populations represent potentially different genetic resources (Manzi & Hadley 1991, Newkirk & Waugh 1977, Newkirk 1978, Mallet & Haley 1983, Stiles 1978, Bower & Blackburn 1997, Cruz & Ibarra 1997, Chevassus 1983).

The purpose of this study is to investigate population differences and heterosis in growth traits and survival among three samples taken from Chinese, Japanese and Korean populations of the Zhikong scallop, *Chlamys farreri*. The population samples were mated to produce pure and reciprocal cross progeny.

MATERIALS AND METHODS

Collection and Maintenance

The sample of the Chinese scallop (CC) was collected from the cultured population in Dalian Bay, Dalian City, Liaoning province (north latitude 38°42'N, east longitude 121°31'E, and annual temperature range 1.0–22.8°C). The sample of the Korean scallop (KW) was collected from a wild population on the sea coast of Korea (north latitude 35°28'N, east longitude 130°28'E, and annual temperature range 4.5–23.7°C). The sample of the Japanese scallop (JW), also from a wild population, was collected from the Pacific seacoast of Japan (north latitude 40°52'N, east longitude 142°22'E, annual temperature range 2.5–21.6°C. Each sample was

This is contribution number G199901209 of 973 from the Chinese basic research project.

*Corresponding author. E-mail: jhxiang@ms.qdio.ac.cn

held for half year in a maturation unit prior to mating. The diet consisted of fresh *Isochrysis zhanjiangensis* and *Platymonas* sp.

Experimental Design and Cross-fertilization

Fertilization between and within the three population samples were conducted at room temperature (19–21°C): CC male × CC female, JW male × CC female, KW male × CC female, CC male × JW female, JW male × JW female, KW male × JW female. Only male scallops were included in the sample taken from the KW population. Thus, the within and cross genotype fertilizations involving KW females are not included in this study because of no female spawning eggs.

A random sample of 80–100 adult *Chlamys farreri* individuals (7–10 cm length) from each of the three populations were taken for spawning. Each sample was dried in the shade for 2 hours and placed in a separate container filled with UV-sterilized filtered seawater to collect sperm and ovum. Fertilization was accomplished through mixing of the diluted sperm and diluted ovum containers. After 2–4 hours the sperm-ovum mixture was rinsed in seawater two to three times and incubated in seawater in 100-L plastic tanks at room temperature.

Larval and Juvenile Rearing

Larvae were reared on a diet of mixed algae, *Isochrysis zhanjiangensis* and *Platymonas* sp. All cultures were carried out in filtered seawater (FSW) at 19–23°C, the approximate ambient seawater temperature. Each day 50% to 75% of the culture water was removed by reverse filtration/siphoning and replaced with fresh FSW. Fresh air was continuously pumped into each culture.

Culture of Adults

After 2 months of age the cultured juveniles from each group were placed separately into plastic cages and suspended in the sea. Body weight and shell height and width and length measurements were recorded every 2–3 months, when the cages were changed, until 24 months of age.

Measurement Date

Although the experimental design called for weight and linear measurements to be recorded at 60-day intervals, weather conditions and workload sometimes precluded doing this.

Measurement and Statistical Analyses

From each progeny subclass 100–150 offspring were sampled for measurements of body size (shell length, S-L; shell height, S-H; shell width, S-W) and body weight (B-W) at approximately 2-month intervals. Within each test period the percent heterosis (H) of each cross was computed as:

$$H(\%) = \frac{F_1 - \frac{1}{2}(P_1 + P_2)}{\frac{1}{2}(P_1 + P_2)} \times 100 \quad (1)$$

where, F_1 , P_1 , P_2 represent trait average of the first generation cross, parent 1 and parent 2, respectively. Incremental change (G) in body weight and in each linear measurement for each period was computed as:

$$G = (W_2 - W_1)/(t_2 - t_1), \quad (2)$$

where t_1 and t_2 represent time at the beginning and end of each period and W_1 and W_2 represent the menstruation value at the beginning and end of each period.

RESULTS

Comparison of Heterosis of Different Crossbred for Different Characters in Each Period

The average and standard deviation of each progeny group for body weight and linear measurement, including calculated heterosis for the reciprocal crossbred samples CC × JW and JW × CC, for each test period are included in Table 1. The Chinese cultured population has similar body weight growth, as does the Japanese wild population. Across the nine measurement intervals the F_1 hybrid progeny of three *Chlamys farreri* samples exhibited a wide range of heterosis, from 4% to 50% (see Table 1). Among the F_1 progeny the JW × KW cross exhibited the most heterosis followed by the CC × KW, JW × CC, and CC × JW crosses. Among the growth traits there were distinct differences in the magnitude of heterosis expression. Shell height tended to be more heterotic than shell length followed by shell width and wet weight. The expression of heterosis in each trait tended to decrease as age of the scallop increased.

In this study the F_1 hybrid combinations exhibited satisfactory levels of heterosis over the nine evaluation periods. The JW female × KW male and CC female × KW male F_1 were the optimal crosses, than can be applied in aquaculture production.

Comparison of the Growth Rate for Four Hybrids and the Purebred

The means (δ , ccm/d, and mg/d) for growth rate of shell length, width, and height and for body weight by age in months for the CC and JW parental samples and four F_1 progeny samples are included in Figure 1.

Growth rate for each trait in each sample reached its pinnacle at 13 months of age. The incremental changes in growth were greater for each trait in each hybrid sample than in the pure parental samples.

The Growth Trend of Chlamys Farreri for Different Characters for Four Hybrids and the Offspring of Pure Parents by Age in Months

Based on the serial measurements of wet weight and shell length increase in parental and F_1 sample of *Chlamys farreri*, growth differences among the parental and F_1 progeny samples were evaluated (Fig. 2).

The growth in shell length tends to plateau at approximately 16 months of age. The growth curve for both wet weight and shell length was greater in the F_1 progeny samples than in the pure parental samples. Among the F_1 progeny samples, the growth curve for wet weight and shell length tended to be different.

Comparison of Growth at 24 Months of Age for Three Hybrids and the Offspring of Their Parental Populations

At 24 months of age the wet weight of the F_1 progeny samples was heavier than their pure parental samples (see Fig. 2 A). Much of the difference between the F_1 progeny and their parental pure counterparts can be attributed to heterosis. Wet weight is the most important trait in scallop production and hybridization can be a useful tool in improving wet weight growth. However, not all F_1 combinations result in equal growth in wet weight. Therefore, experimental evaluation of crosses between sampled populations

TABLE 1.

Comparison of survival and heterosis of growth of hybrids with their parental populations. Values marked with different superscripts are significantly different from each other at 0.05.

Age	Combination Character/ Index	CC X ± S	F ₁ (CC × JW)		F ₁ (JW × CC)		JW X ± S	F ₁	F ₁
			X ± S	Heterosis (%)	X ± S	Heterosis (%)		(JW × KW) X ± S	(CC × KW) X ± S
Survival (%)		82.9 ^b ± 8.9	90.0 ^a ± 10.2	13.31	87.6 ^a ± 9.6	10.28	75.9 ^c ± 23.2	90.8 ^a ± 11.4	92.1 ^a ± 13.1
5 months	S-L/cm	1.35 ^b ± 0.20	1.75 ^a ± 0.22	30.32	1.81 ^a ± 0.27	34.55	1.34 ^b ± 0.26	1.85 ^a ± 0.31	1.83 ^a ± 0.29
	S-H/cm	1.64 ^b ± 0.24	2.16 ^a ± 0.290	27.86	2.22 ^a ± 0.27	31.95	1.73 ^b ± 0.31	2.20 ^a ± 0.28	2.10 ^a ± 0.24
	S-W/cm	0.44 ^a ± 0.05	0.53 ^b ± 0.09	18.73	0.57 ^a ± 0.07	27.71	0.45 ^c ± 0.10	0.59 ^a ± 0.08	0.57 ^a ± 0.07
	B-W/g	1.23 ^c ± 0.26	1.54 ^b ± 0.43	12.06	1.68 ^a ± 0.59	21.86	1.53 ^b ± 0.40	1.73 ^a ± 0.47	1.70 ^a ± 0.42
	S-L/cm	1.63 ^b ± 0.18	2.12 ^a ± 0.42	30.36	2.18 ^a ± 0.23	34.09	1.62 ^b ± 0.24	2.30 ^a ± 0.22	2.23 ^a ± 0.34
9 months	S-H/cm	1.99 ^a ± 0.23	2.42 ^b ± 0.37	23.05	2.56 ^{ab} ± 0.24	29.95	1.95 ^b ± 0.29	2.72 ^a ± 0.22	2.63 ^b ± 0.30
	S-W/cm	0.53 ^b ± 0.07	0.67 ^a ± 0.11	23.53	0.71 ^a ± 0.07	29.60	0.56 ^b ± 0.17	0.72 ^a ± 0.08	0.71 ^a ± 0.08
	B-W/g	1.49 ^c ± 0.35	1.91 ^b ± 0.79	28.37	2.15 ^a ± 0.61	44.73	1.48 ^c ± 0.43	2.24 ^a ± 0.64	2.18 ^a ± 0.77
	S-L/cm	1.98 ^b ± 0.38	2.39 ^a ± 0.45	18.43	2.42 ^a ± 0.40	19.46	2.06 ^b ± 0.39	2.55 ^a ± 0.40	2.47 ^a ± 0.39
	S-H/cm	2.39 ^b ± 0.44	2.85 ^a ± 0.60	18.77	2.88 ^a ± 0.47	19.90	2.41 ^b ± 0.41	2.96 ^a ± 0.48	2.86 ^a ± 0.42
11 months	S-W/cm	0.68 ^b ± 0.11	0.79 ^a ± 0.13	14.31	0.82 ^a ± 0.12	18.28	0.71 ^b ± 0.12	0.83 ^a ± 0.16	0.83 ^a ± 0.13
	B-W/g	1.93 ^b ± 0.52	2.85 ^a ± 0.53	44.36	2.97 ^a ± 0.53	50.80	2.01 ^b ± 0.52	3.05 ^a ± 0.52	2.97 ^a ± 0.51
	S-L/cm	3.80 ± 0.39	4.29 ± 0.52	10.51	4.53 ± 0.22	16.78	3.95 ± 0.47	4.59 ± 0.56	4.59 ± 0.61
	S-H/cm	4.33 ± 0.36	4.66 ± 0.57	7.12	5.00 ± 0.352	15.03	4.37 ± 0.42	5.23 ± 0.54	5.16 ± 0.54
	S-W/cm	1.34 ^b ± 0.15	1.56 ^a ± 0.16	14.01	1.62 ^a ± 0.11	18.25	1.40 ^b ± 0.14	1.66 ^a ± 0.22	1.65 ^a ± 0.17
13 months	B-W/g	12.55 ^c ± 2.08	16.15 ^b ± 4.03	25.87	17.45 ^{ab} ± 1.68	36.00	13.11 ^c ± 3.02	18.48 ^a ± 4.83	18.29 ^a ± 4.38
	S-L/cm	5.35 ^c ± 0.38	5.76 ^b ± 0.57	8.10	5.94 ^a ± 0.40	11.48	5.32 ^b ± 0.47	5.92 ^a ± 0.53	5.89 ^a ± 0.59
	S-H/cm	5.60 ^b ± 0.42	6.28 ^a ± 0.59	11.56	6.44 ^a ± 0.38	14.40	5.65 ^b ± 0.48	6.49 ^a ± 0.47	6.30 ^a ± 0.56
	S-W/cm	2.00 ^b ± 0.20	2.15 ^{ab} ± 0.20	6.90	2.21 ^a ± 0.15	9.60	2.03 ^b ± 0.20	2.23 ^a ± 0.19	2.12 ^{ab} ± 0.17
	B-W/g	30.63 ^b ± 4.81	38.34 ^a ± 9.26	22.49	39.94 ^a ± 6.60	27.57	31.98 ^b ± 7.42	40.25 ^a ± 9.45	37.62 ^a ± 8.40
16 months	S-L/cm	5.39 ^b ± 0.40	5.72 ^b ± 0.48	5.52	6.01 ^a ± 0.37	10.93	5.44 ^c ± 0.48	6.02 ^a ± 0.43	5.94 ^a ± 0.56
	S-H/cm	5.65 ^b ± 0.38	6.23 ^a ± 0.51	8.73	6.48 ^a ± 0.38	13.17	5.81 ^b ± 0.57	6.51 ^a ± 0.54	6.38 ^a ± 0.52
	S-W/cm	2.01 ± 0.20	2.23 ± 0.24	8.54	2.25 ± 0.16	9.78	2.09 ± 0.21	2.26 ± 0.22	2.14 ± 0.20
	B-W/g	27.70 ^c ± 6.96	34.83 ^b ± 8.21	20.08	38.05 ^a ± 5.85	31.19	30.31 ^c ± 7.83	39.67 ^a ± 0.52	36.8 ^{ab} ± 0.48
	S-L/cm	5.47 ^b ± 0.45	5.92 ^a ± 0.66	7.37	6.11 ^a ± 0.51	10.83	5.56 ^b ± 0.51	6.07 ^a ± 0.44	5.97 ^a ± 0.53
18 months	S-H/cm	5.79 ^b ± 0.43	6.40 ^a ± 0.70	10.08	6.50 ^a ± 0.55	11.82	5.84 ^b ± 0.76	6.52 ^a ± 0.56	6.43 ^a ± 0.46
	S-W/cm	2.05 ± 0.23	2.23 ± 0.56	7.14	2.26 ± 0.19	8.48	2.11 ± 0.25	2.26 ± 0.79	2.15 ± 0.19
	B-W/g	30.98 ^b ± 9.50	38.40 ^a ± 9.50	23.43	39.59 ^a ± 8.99	27.17	31.28 ^b ± 7.59	41.34 ^a ± 8.72	40.35 ^a ± 7.06
	S-L/cm	5.54 ^b ± 0.49	6.00 ^a ± 0.52	6.40	6.15 ^a ± 0.55	9.17	5.73 ^b ± 0.51	6.13 ^a ± 0.49	6.10 ^a ± 0.54
	S-H/cm	6.10 ^b ± 0.48	6.51 ^a ± 0.52	6.67	6.53 ^a ± 0.54	7.06	6.09 ^b ± 0.63	6.62 ^a ± 0.48	6.52 ^a ± 0.39
20 months	S-W/cm	2.08 ± 0.21	2.22 ± 0.19	4.59	2.30 ± 0.45	8.40	2.17 ± 0.22	2.35 ± 0.65	2.26 ± 0.17
	B-W/g	36.15 ^c ± 8.03	40.40 ^b ± 9.50	10.52	41.89 ^{ab} ± 10.1	14.59	36.97 ^c ± 8.94	43.34 ^a ± 5.84	42.54 ^a ± 6.12
	S-L/cm	5.81 ^b ± 0.53	6.14 ^a ± 0.47	4.93	6.18 ^a ± 0.44	5.71	5.90 ^b ± 0.52	6.21 ^a ± 0.51	6.16 ^a ± 0.48
	S-H/cm	6.42 ^b ± 0.54	6.69 ^a ± 0.46	4.84	6.67 ^a ± 0.45	4.42	6.34 ^b ± 0.49	6.71 ^a ± 0.51	6.65 ^a ± 0.42
	S-W/cm	2.24 ^b ± 0.20	2.41 ^a ± 0.31	7.32	2.45 ^a ± 0.21	9.42	2.24 ^b ± 0.20	2.43 ^a ± 0.19	2.42 ^a ± 0.19
22 months	B-W/g	39.32 ^c ± 8.32	44.8 ^b ± 9.98	12.15	45.26 ^b ± 9.25	13.19	40.65 ^c ± 10.29	48.48 ^a ± 9.58	46.49 ^{ab} ± 7.7

becomes a necessary precondition to include hybridization of populations in commercial scallop culturing. Figure 2 B includes the differences at 24 months of age among the F₁ and parental pure progeny samples for shell length. The shell length of the F₁ samples was much greater than those of the parental populations. As was the case for wet weight, heterosis was an important part of the increase in shell length in the F₁ progeny samples.

DISCUSSION

Comparison of the Survival for Four Hybrids and the Offspring of Two Parent Populations

The percentage of survival from the zygotes to juveniles of 2 months of age for four hybrids and the offspring of two parent populations were calculated in Table 1. The percentage of survival for four hybrids were significantly greater than the purebred ($P < 0.05$), and the percentage of survival for Chinese purebred was

significantly greater than that of Japanese purebred, there were no significant differences of survival percentage among the four ($P > 0.05$).

Comparison of the Growth for Three Hybrids and the Offspring of Three Parent Populations

Our evaluation of four phenotypic traits, shell length, shell width, shell height and body weight, suggest genetic differences among two scallop populations and their reciprocal F₁ crosses evaluated simultaneously under the same environmental conditions. From age 5 months to 24 months, there were differences in body weight and shell linear sizes over the sampling period. The samples from the Chinese cultured population and the Japanese wild population were similar, but crossbred offspring were consistently faster ($P < 0.05$) growing than purebred offspring (see Table 1).

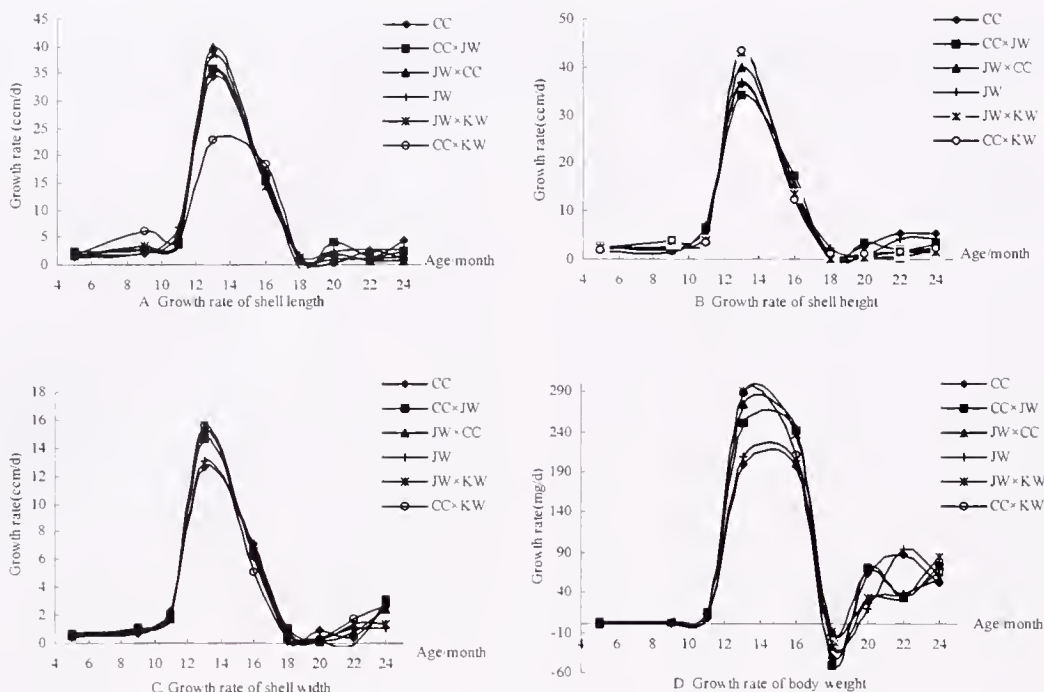


Figure 1. Comparison of growth rate of four characters for four hybrid stocks and their parental populations, (A) growth rate of shell length; (B) growth rate of shell height; (C) growth rate of shell width; and (D) growth rate of body weight.

Comparison of the Growth Among the Different Periods

The growth rates for each of the four phenotypic traits included in this study are influenced by seasonal environmental conditions. During winter months the cold water causes the growth rate of scallops to approach zero or even lose body weight and size. However, during the warmer seasons of summer and fall growth rate rapidly increases.

Comparison Among the Growth Rate of Four Phenotypic Traits Over the Evaluation Period

The growth rates of each of the four phenotypic traits, shell length, shell width, shell height, and body weight tend to be different. Among these traits body weight growth is the fastest fol-

lowed by shell length, shell width, and shell height. Therefore, among the linear shell measurements the shell length should be the most important selection trait in a breeding program.

Breeding and Heterosis Utilization of *Chlamys Farreri*

Scallop breeding has received considerable research attention because the development of aquaculture has increased. Developments in artificial insemination and juvenile rearing techniques have succeeded in improving the efficiency of scallop culturing. However, performance of the economic traits and purity of the cultural stocks have been reduced because of the idioplasm retrogression and the parental scallops are wild populations. Thus, it is difficult to expect stable levels of heterosis. Selective breeding and evaluation of new breeds and strains are the bases of the hybridization breeding programs. Such breeding programs can provide

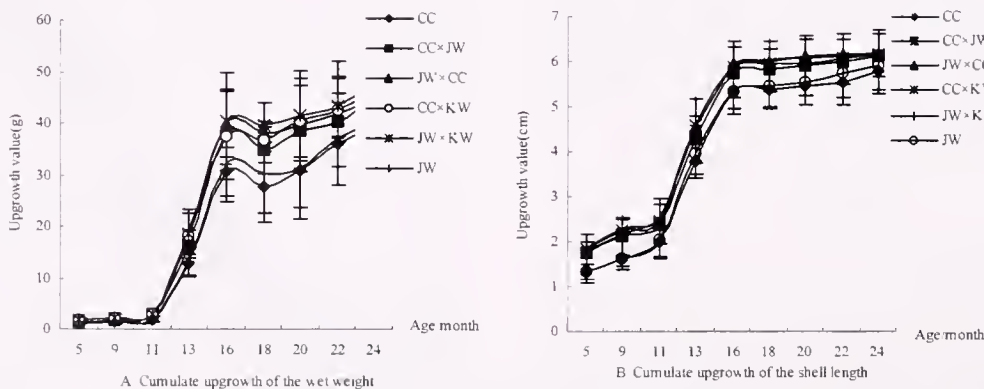


Figure 2. Cumulate growth comparison of Chinese and Japanese populations with four populations of hybrids, (A) cumulate growth of body weight and (B) cumulate growth of shell length.

pure hybridization parents and overcome the idioplasm retrogression. The research reported here shows that for wet shell weight and linear shell measurements in crosses among cultured and wild

populations of the *Chlamys farreri* scallop, positive heterosis are expressed at levels sufficient to warrant systematic hybridization in the culture of scallops.

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LIPID CLASSES AND FATTY ACID COMPOSITION IN FEMALE GONADS OF GREAT SCALLOPS—A SELECTIVE FIELD STUDY

ARNE DUINKER,* BENTE E. TORSTENSEN, AND OYVIND LIE

National Institute of Nutrition and Seafood Research, PO Box 176 Sentrum, 5804 Bergen, Norway

ABSTRACT Fatty acid composition was analyzed in neutral lipids (NL), phosphatidyletanolamine (PE) and phosphatidylcholine (PC) of *Pecten maximus* (L.) ovaries from Western Norway. Individual ovaries were selected based on macroscopic characterization. Two series were compared; (1) ovary development from recovering to half-full ovaries during the autumnal restoration of the gonads and (2) seasonal variation with late filling ovaries from different parts of the year. The relative lipid content and the proportion of triacylglycerols increased during development. Changes in fatty acid composition during ovary development were minor compared with the changes observed with seasonal variation. The samples from June differed from the other samples with a large increase in 20:5n-3 on the expense of 22:6n-3 in neutral lipids (NL) and phosphatidylcholine (PC) and a concomitant increase in 20:5n-3 and 22:6n-3 in phosphatidyletanolamine (PE). The changes in NL and PC can probably be attributed to a dietary effect from the diatom dominated spring phytoplankton bloom. Only small changes in the degree of unsaturation in PC were seen that may be due to a temperature effect. The differences observed represent large differences in status of broodstock from field prior to conditioning in season independent hatchery production.

KEY WORDS Fatty acids, lipid composition, ovaries, *Pecten maximus*, phospholipids, scallops, triacylglycerols

INTRODUCTION

The importance of lipids for the quality of eggs and larvae of bivalves is well established. Broodstock diet in particular influences the lipid composition in the gonads and eggs (Helm et al. 1991, Utting & Doyou 1992, Soudant et al. 1996a, Soudant et al. 1996b, Soudant et al. 1996c, Soudant et al. 1999). Special attention has been given to the polyunsaturated fatty acids 22:6n-3, 20:4n-6 and to a lesser but still important extent 20:5n-3. These are selectively incorporated in the phospholipids during gonad build up, despite deficiencies in diet (Soudant et al. 1996a). Lipid composition of the ovaries and eggs then affects larval performance. 22:6n-3 has been demonstrated as essential for growth and survival of bivalves (Langdon & Waldo 1981). Higher levels of 22:6n-3 and a higher 22:6n-3/20:5n-3 ratio were associated with improved hatching rate and enhanced gametogenesis in *Pecten maximus* (Soudant et al. 1996a, Soudant et al. 1996b), while growth rate was significantly related to the content of 22:6n-3 in oyster larvae (Thompson & Harrison 1992, Berntsson et al. 1997). Another factor known to influence membrane lipid composition in poikilotherms is temperature. Lipid class and fatty acid composition is regulated according to the temperature to maintain optimal fluidity of the membranes (Williams & Hazel 1994), and dietary input of essential fatty acids may be required for cold acclimation (Farkas et al. 1980). Increased unsaturation of the phospholipids or increase in sterol content at low temperatures is also reported for bivalves (Ueda 1974, Piretti et al. 1988, Chu & Greaves 1991, Napolitano et al. 1992).

Seasonal changes in lipid composition of ovaries have been reported in several studies of scallops (Besnard et al. 1989, Napolitano & Ackman 1992, Pazos et al. 1997). Changes in lipid composition have been related to reproductive activity, but the effects of season and stage of maturity have not been separated. Scallops in the field experience large fluctuations in food composition from various phytoplankton blooms, known to differ in fatty acid composition (Budge et al. 2001) which gives imprints in filter feeders (Pedersen et al. 1999, Shirai et al. 2002). Temperature

fluctuations in the field can also affect lipid composition of bivalves (Ueda 1974, Piretti et al. 1988). In seasonal independent hatchery production of *P. maximus* in Norway, broodstock animals are taken from the sea for conditioning in the hatchery at different times of the year. Variations in lipid composition of animals from the field will hence represent differences in status prior to conditioning.

Field data are often important basis for evaluating the quality of artificial conditioning and other aquacultural manipulations (e.g. Soudant et al. 1999). A study was performed to describe variations in lipid composition in ovaries from a *P. maximus* field population. From a parallel study, large variations in ovary developmental stages were seen during the reproductive cycle of this population (Duinker & Nylund 2002). Individual ovaries were therefore selected based on macroscopic characteristics of ovary development, to get a more precise description of lipid composition in the different ovary developmental stages than a population average would have given. Two series of ovaries were selected, one consisting of ovaries in the same stage of development from different times of the year, and one with successive developmental stages during the autumn restoration of the gonads. The aim of this study is to compare seasonal variations in lipid class and fatty acid composition with the variations during development.

MATERIALS AND METHODS

Sampling Procedures

Individual ovaries were sampled monthly in combination with studies of seasonal variation in chemical composition of ovaries and storage organs in *Pecten maximus* (Strohmeier et al. 2000) and histologic and visual characterizations of the ovaries (Duinker & Nylund 2002). The scallops were collected in Raunefjorden south of Bergen, Norway, and ovary samples were stored at -80°C . Ovaries were selected based on visual characterization as described by Duinker & Nylund (2002). The characterization is based on the study of Mason (1958), and the stages sampled for ovary development were (numerical values in brackets): "recovering" (3), "filling" (4), "late filling" (4.5), and "half full" (5). These ovaries were sampled on September 15, October 18, December 12, 1997, and February 7, 1998, respectively (Table 1).

*Corresponding author. Email: Duinker@nifes.no

TABLE 1.

Visualisation of the sampling dates for the ovary development series and the samples for seasonal variation.

	Ovary development	Ovary development (spring control)	Seasonal variation
Jan		4	
Feb		5	
Mar			4.5
Apr			
May			
Jun			4.5
Jul			
Aug			
Sep	3		
Oct	4		
Nov			
1997 Dec	4.5		4.5
1998 Jan			
Feb	5		4.5

The numbers represent the ovary development stages recovering, (3); filling, (4); late filling, (4.5); and half full, (5).

Filling (4) ovaries from February 15, 1997 and half-full (5) ovaries from March 12, 1997 were sampled as spring controls for comparison with the corresponding stages sampled during autumn. Only changes that were consistent between the samples in both series were considered as related to ovary development. Late filling (4.5) ovaries were selected for seasonal variation from the March 31, June 8, December 12, 1997, and February 7, 1998 samples. The samples for the late filling stage in the ovary development series and the seasonal variation sample from December were identical.

Analytical Procedures

The ovary samples were freeze-dried and dry weight percentage was determined. After homogenizing the samples, lipids were

extracted with chloroform-methanol (2:1, v/v), modified from Bligh and Dyer (1959) according to Ronnestad et al. (1995), and total lipid content was determined gravimetrically. Further, lipid classes were separated by HPLC as described by Lie and Lambertsen (1991) using a Constametric II solvent-delivery system at 205 nm with a variable-wavelength spectrophotometer (LDC Spectromonitor III). The column (25 cm \times 0.46 cm I.D.) was packed with silica gel (LiChrosorb 5 μ m, Merck). The initial solvent mixture consisted of hexane:2-propanol:acetonitrile (4:3:7, v/v/v). This was added to the protocol of Lie & Lambertsen (1991) and eluted two unidentified peaks¹, whereas NL eluted with the front. The second solvent mixture of hexane:2-propanol:acetonitrile: water (372:496:97:35, by volume) eluted PE. PC was eluted with hexane-2-propanol-water (394:526:80, v/v/v). The identity of the isolated phospholipids was checked by TLC (Kiesel-gel 60 Merck), using phospholipid standards and a solvent system of ethyl acetate-n-propanol-chloroform-methanol-0.25% aqueous KCl (15:25:25:10:4.5, by volume).

The three fractions were collected manually into vials, saponified, and esterified in 12% BF₃ in methanol, and the fatty acid composition of the different lipid classes was determined according to Lie and Lambertsen (1991). The methyl esters were separated using a Carlo Erba gas chromatograph ("cold on column" injection, 60°C for 1 min at 25°C min⁻¹, 160°C for 28 min at 25°C min⁻¹, 190°C for 17 min at 25°C min⁻¹, 220°C for 2 min), equipped with a 50-m CP-sil 88 (Chromopack) fused silica capillary column (i.d. 0.32 mm, 0.20- μ m film) (Lie & Lambertsen 1991). The fatty acid composition was calculated using an integrator (Turbochrom Navigator, Version 6.1), connected to the GC and identification ascertained by standard mixtures of methyl esters (Nu-Chek, Elyian, USA). The quantification of lipid classes in

¹One peak was rich in 22:6n-3 and the other was rich in 20:4n-6. These were eluted similarly as the fraction earlier assumed to be a glycolipid (Soudant et al. 1995), now identified as cardiolipin (Kraffe et al. 2002). The two peaks were only separable using new columns in the present set-up. They were not identified and are thus not reported here.

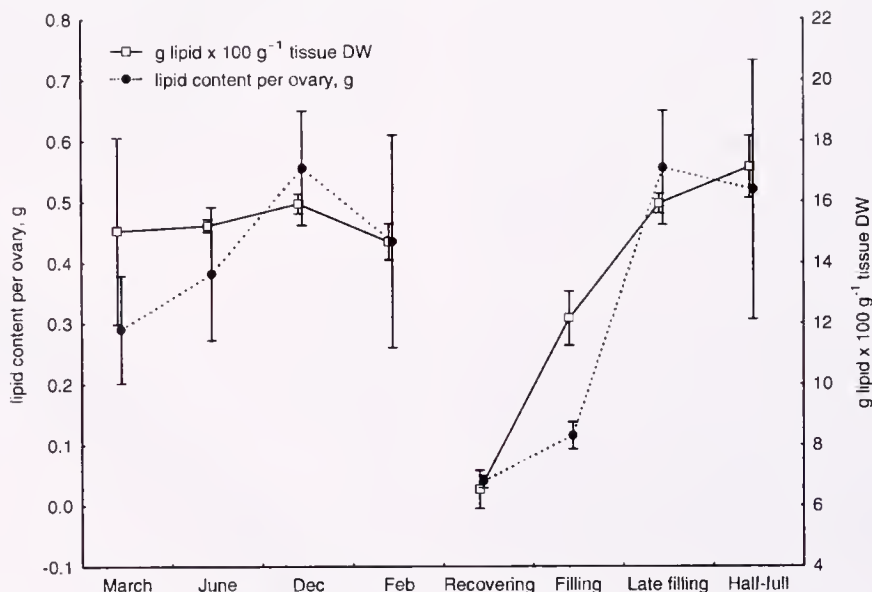


Figure 1. Lipid content (g) per ovary (dotted line, left axis) and mg lipid per 100 g tissue dry weight (solid line, right axis). Vertical bars indicate standard deviations, $n = 3$.

recovering (3) and half-full (5) ovaries was performed using high-performance thin-layer chromatography (HPTLC) as described by Bell et al. (1993). Total lipid (10 µg) was applied to a 10 × 20 cm HPTLC plate that had been prerun in hexane:diethyl ether (1:1 v/v) and activated at 110°C for 30 min. The plates were developed at 5.5 cm in methyl acetate: isopropanol: chloroform: methanol: 0.25% (w/v) aqueous KCl (25:25:25:10:9, by volume) to separate phospholipid classes with neutral lipids running at the solvent front (Vitello & Zanetta 1978). After drying, the plates were developed fully in hexane: diethyl ether: acetic acid (80:20:2, v/v/v) to separate neutral lipids and cholesterol. Lipid classes were visualized by charring at 160°C for 15 min after spraying with 3% copper acetate (w/v) in 8% (v/v) phosphoric acid identified by comparison with commercially available standards. Lipid classes were quantified by scanning densitometry using a CAMAG TLC Scanner 3 and calculated using an integrator (WinCATS-Planar Chromatography, Version 1.2.0).

Statistics

The data for fatty acid composition in the lipid classes of recovering (3) and half-full (5) ovaries were compared using the Mann-Whitney test (Zar 1999). With the low *n* of three individuals per point these statistics are only used as guidance for interpreting the results. The relative fatty acid composition data of the lipid classes was analyzed using SIRIUS for Windows (Pattern recognition systems AS, Norway, Version 6.0). Principal component analysis (PCA) (Wold et al. 1987) was performed in the data matrix of the relative fatty acid compositions. The purpose of PCA is to express the main information in the variables by a lower number of variables, the so-called principal components (PC1, PC2, ...). A high positive or negative loading reveals a significant variable in the actual PCA model. Score plots from the PCA explore the main trends in the data, and their respective loading reveal fatty acids with a significant loading. Basically, samples or variables with a similar angle from origin in the score and loading plots, respectively, are positively correlated. A 90° angle reflects no correlation and towards 180° reflects negative correlation. Variables (fatty acids) and samples can be compared in the same way, so that samples are positively influenced by variables with a similar angle from origin and so on.

RESULTS

The relative lipid content of dry weight in the ovaries increased linearly 2.6-fold during development from recovering (3) to half-full (5) ovaries. In contrast, there was no variation in relative lipid content throughout the season (Fig. 1). Total content of lipid per ovary increased 10-fold during development, whereas only some variation due to different sizes of the gonads were seen in the samples for seasonal variation, with no clear trends (see Fig. 1). Triacylglycerols (TAG) increased from 32% in recovering ovaries to 47% in half-full ovaries concomitant with a corresponding decrease in phospholipids (PL) and cholesterol (Fig. 2a). This corresponds to an increase in neutral lipids from 46% to 61% if only fatty acid containing classes are quantified, hence omitting cholesterol and adding up TAG and free fatty acids (FFA). Within the PL, only marginal differences were seen when comparing recovering (3) and half-full (5) ovaries (see Fig. 2b).

Multivariate principal component analysis (PCA) of the relative fatty acid composition of the neutral lipids (NL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) showed that

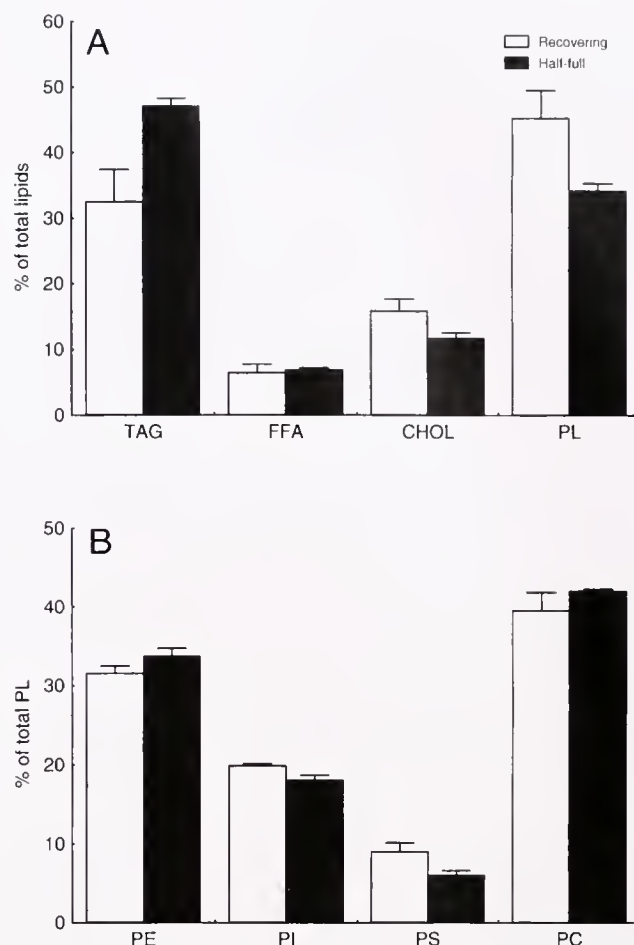


Figure 2. Relative lipid class composition in recovering (3) and half-full (5) ovaries sampled September 15, 1997 and February 7, 1998 respectively. A. Neutral lipids (TAG, triacylglycerids, FFA, free fatty acids; CHOL, cholesterol) vs. total phospholipids (PL) as percent of total lipids. B. The phospholipid classes phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylcholine (PC), as percent of total phospholipids. Vertical bars indicate standard deviations, *n* = 3.

each of the lipid classes analyzed has its characteristic fatty acid composition and are separated in the score plot (Fig. 3). The variables 22:6*n*-3 and 16:0 had high positive loadings along PC2, whereas 18:0 and 20:5*n*-3 had high negative loadings along PC2. Further, 22:6*n*-3 and 18:0 had high negative loadings along PC1, and 16:0 and 18:4*n*-3 had high positive loadings along PC1. These loadings correspond to the grouping of the PC, PE, and NL samples with PC containing high levels of 22:6*n*-3 followed by PE and NL. Furthermore, NL contained high levels of 18:4*n*-3 and lower levels of 22:6*n*-3 compared with the phospholipid classes. The level of 20:5*n*-3 was highest in PE; 16:0 was the dominant saturated fatty acid in PC whereas 18:0 was dominant in PE (see Fig. 3). During development, 20:5*n*-3 increased 1.6 fold, 1.5-fold and 1.9-fold in NL, PE and PC, respectively (Fig. 4 and Table 2). The main increase was between the recovering (3) and filling (4) stages. During development, 22:6*n*-3 decreased slightly in NL, increased 1.3 fold in PE and showed no variance of significance in PC. The largest differences in fatty acid composition, however, were seen in ovaries from June compared with the other samples for seasonal variation. In June 20:5*n*-3 was markedly higher on the

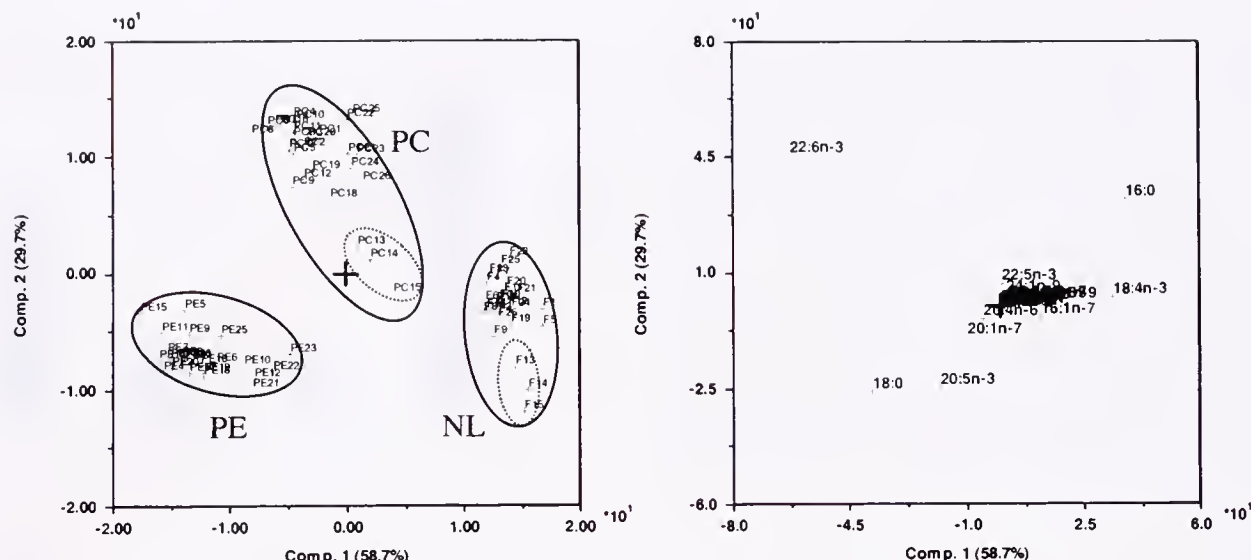


Figure 3. Separation of the different lipid classes (encircled by hand) according to their fatty acid composition using multivariate principal component analysis. The samples from June are encircled with a dotted line in PC and NL.

expense of 22:6n-3 in NL and PC (see Fig. 4), while both 20:5n-3 and 22:6n-3 were higher in PE. This shift resulted in a decrease in the 22:6n-3/20:5n-3 ratio between the other samples and June from 2.7 to 1 in PC and 1.2 to 0.5 in NL, while the ratio was relatively stable in PE.

The ratio of polyunsaturated/saturated fatty acids showed some differences with season in PC, though values from December overlapped with values from February. The ratio was higher in March (3.1) and February (2.8), compared with June (2.6) and December (2.5). The concurrent increase in 20:5n-3 and 22:6n-3 in June lead to higher ratio of polyunsaturated/saturated fatty acids in PE. Some minor changes in this ratio in NL were not consistent with the changes in PC or PE. In NL, the ratio of 16:1n-7/16:0 was higher in June (0.38) compared with the other samples for seasonal variation (0.15–0.25), and also 16:4n-3 was highest in June.

Dimethylacetals (DMA) from PE plasmalogens in the GC analyses were not identified. However, possible candidates with retention times slightly shorter than 18:0 (pers. comm. Y. Marty), never exceeded 5% of total fatty acids. Analysis of selected PE methyl ester fractions from the present study by Y. Marty (University of Brest, France) showed similar low content of DMA. Discrepancy with Soudant et al. (1995) and later works reporting higher levels of DMA may be attributed to incomplete derivation of alkenyl chains into DMA or differences between the populations.

DISCUSSION

Small changes were seen in the relative amounts of lipid classes and their respective fatty acid composition during development from recovering (3) to half-full (5) ovaries. This is consistent with the cellular changes during ovary development in *P. maximus*. In developing ovaries there is a discontinuous but ongoing recruitment of new cohorts of oocytes. A gradual accumulation of ripe oocytes is seen, with multiple cohorts of different development stages present at the same time (Paulet & Boucher 1991). Hence, changes in composition during ovary development have limited relation to individual oocyte development, particularly during the earliest stages. In empty ovaries (stage 2), the connective tissue may have a substantial contribution to the lipid content relative to

the young oocytes. Recovering (3) ovaries may have different cellular composition according to their history (Duinker & Nylund 2002). The recovering ovaries in the present study, more correctly termed as "almost spent", contained relatively few young oocytes in a mixture with developing and fully-grown oocytes (Duinker unpublished data). "Truly" recovering ovaries with numerous early vitellogenic oocytes in active development would better represent early oocyte development, but such ovaries were not found among the samples from September and October. The further stages of ovary development from filling (4) to half-full (5) also contain mixtures of several cohorts developing at the same time, but with increased accumulation of ripe oocytes. Hence, the changes in lipid composition during this development reflect a gradual increase in the proportion of ripe oocytes. If oocyte development had been a primary interest of the study, a size grading of oocytes after separation from the connective tissue would be a better approach. However, in this study the ovary development itself is of primary interest in comparison with seasonal changes.

During development from recovering (3) to half full (5) ovaries, both relative lipid composition and the total content per ovary increased concomitant with a corresponding increase in all lipid classes during the 10-fold increase in total lipid content. Triacylglycerols (TAG) was the dominant lipid class and increased from about 32% to 47% of total lipids between recovering (3) and half-full (5) ovaries. The levels are comparable with other studies of scallops (Napolitano & Ackman 1992, Soudant et al. 1996c, Pazos et al. 1997). Increase in neutral lipids (NL) is found both during conditioning (Soudant et al. 1996a) and with maximum maturity in other field studies (Besnard et al. 1989, Napolitano & Ackman 1992, Pazos et al. 1997). This probably reflects the increased relative incorporation of NL in the later phases of oocyte development. NL are generally used as energy reserve in all life forms (Sargent 1976), and Gallagher et al. (1986) found a strong decline in NL during embryogenesis prior to the onset of feeding in the larvae of *Crassostrea virginica*, *Ostrea edulis*, and *Mercuria mercenaria*. However, also phospholipids (PL) were utilized in the latter study with some difference among the bivalve species, and Delaunay et al. (1992) suggested an energetic role of PL in

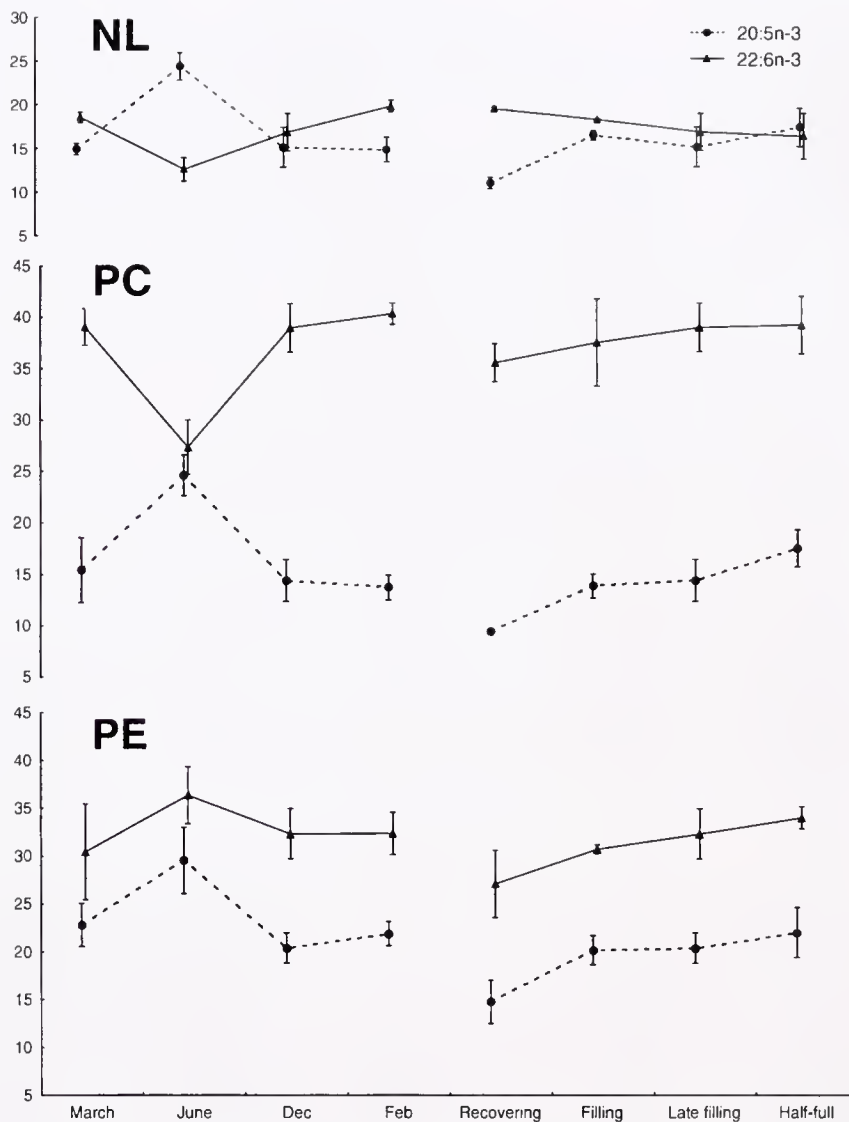


Figure 4. Relative content of 20:5n-3 and 22:6n-3 in the major lipid classes, neutral lipids (NL), phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Vertical bars indicate standard deviations, $n = 3$. Recovering, filling, late filling and half-full ovaries were sampled on September 15, October 18, December 12, 1997 and February 7, 1998 respectively.

P. maximus larvae. Within the PL, the small changes in relative amounts of the different classes during ovary development largely agree with Soudant et al. (1996c). However, these authors found an increase in PC from 29% to 43% on the expense of plasmalogens during conditioning of *P. maximus*, mainly from start to first sampling after 5 weeks of conditioning. These gonads had been emptied by induced spawning at start of the experiment, however, and were hence in an earlier stage at the start of the experiment than the recovering ovaries in the present study. Also in the fatty acid composition small changes were seen during ovary development, in agreement with Soudant et al. (1996c).

The changes in fatty acid composition during ovary development were minor, however, compared with the differences seen in the samples for seasonal variation. The samples from June differed from the samples in February, March, and December, with a marked increase in 20:5n-3 on the expense of 22:6n-3 in PC and NL. One of the major events between March and June was the spring bloom, followed by the spring increase in temperature about

a month later (Strohmeier et al. 2000). The spring bloom in the study area is dominated by the diatom *Skeletonema costatum*, whereas different dinoflagellates and coccolithophorids dominate the rest of the year with only short incidents of diatom blooms (Erga & Heimdal 1984, B.R. Heimdal and A.B. Reisegg pers. comm. 1996). From analyses of algae cultures it is seen that diatoms tend to be rich in 20:5n-3 and relatively deficient in 22:6n-3, whereas various flagellates tend to be rich in 22:6n-3 and relatively deficient in 20:5n-3 (Ackman & Tocher 1968, Chuecas & Riley 1969, Volkman et al. 1981, Ackman 1983, Servel et al. 1994, Dunstan et al. 1994, Berge et al. 1995, Bell & Pond 1996, Soudant et al. 1996a). Large differences are seen among species, but the generalization is supported by analyses of plankton communities and in imprints in herbivores. With changes in dominance of diatoms and flagellates, Budge et al. (2001) found highest levels of 22:6n-3 in seston associated with flagellate dominance, and imprints of the two different types of phytoplankton communities have been reported in zooplankton (Pedersen et al. 1999, Shirai et

TABLE 2.
Relative composition of fatty acids

	NL		PE		PC	
	Recovering	Half-full	Recovering	Half-full	Recovering	Half-full
14:0	4.5 ± 0.3	3.3 ± 0.2*	0.4 ± 0.1	— ± —*	3.5 ± 0.5	1.1 ± 0.3*
15:0	1.0 ± 0.1	0.8 ± 0.1*	2.3 ± 2.0	0.2 ± —	1.4 ± 0.2	0.8 ± 0.1*
16:0	18.2 ± 0.4	16.1 ± 2.0	4.5 ± 1.1	4.7 ± 0.5	19.1 ± 0.8	15.1 ± 1.1*
17:0	3.1 ± 0.2	1.7 ± 0.2*	1.8 ± 0.5	1.7 ± 0.1	1.6 ± 0.1	1.1 ± —*
18:0	3.1 ± 0.4	2.2 ± 0.3*	12.8 ± 2.7	14.1 ± 1.8	3.5 ± 0.8	3.7 ± 0.2
Sum saturated	30.0 ± 0.1	24.1 ± 2.2*	21.9 ± 4.4	20.8 ± 2.2	29.2 ± 1.3	21.8 ± 1.6*
16:1n-7	2.5 ± 1.3	4.7 ± 0.3*	0.3 ± —	0.4 ± —*	1.6 ± 0.2	1.2 ± 0.2
16:1n-9	1.8 ± 1.5	0.9 ± 0.1	— ± —	— ± —	0.8 ± —	0.3 ± —
18:1n-7	3.6 ± 0.3	3.9 ± 0.4	0.7 ± —	0.5 ± —*	2.8 ± 0.4	2.1 ± 0.2*
18:1n-9	3.2 ± 0.2	5.3 ± 0.2*	1.1 ± 0.5	0.6 ± 0.3	2.1 ± 0.4	2.1 ± 0.1
20:1n-7	0.5 ± 0.1	0.4 ± —	— ± —	— ± —	0.1 ± 0.1	— ± —
20:1n-9	0.9 ± 0.2	0.8 ± 0.1	3.2 ± 0.6	0.7 ± 0.1*	0.5 ± 0.1	0.6 ± —*
20:1n-11	0.2 ± 0.1	0.3 ± —	— ± —	— ± —	— ± —	— ± —
22:1n-9	— ± —	— ± —	1.3 ± 2.0	— ± —	— ± —	— ± —
22:1n-11	— ± —	— ± —	0.3 ± 0.5	0.1 ± 0.2	— ± —	— ± —
Sum monoenes	12.6 ± 1.0	16.3 ± 0.9*	6.8 ± 2.6	2.3 ± 0.4*	7.1 ± 1.1	6.3 ± 0.3
18:2n-6	1.8 ± 0.1	2.2 ± 0.2*	0.2 ± 0.2	0.1 ± 0.2	1.0 ± 0.1	0.9 ± —
20:2n-6	0.6 ± 0.1	0.8 ± 0.2	— ± —	0.1 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
20:4n-6	1.3 ± 0.4	1.3 ± 0.2	3.3 ± 0.9	2.7 ± 0.5	1.3 ± 0.4	1.3 ± 0.2
Sum n-6	3.8 ± 0.4	4.3 ± 0.2	3.5 ± 0.9	2.8 ± 0.3	2.8 ± 0.4	2.7 ± 0.1
16:3n-3	0.3 ± 0.1	0.1 ± 0.1	1.2 ± 1.1	0.6 ± 0.2	— ± —	0.2 ± 0.1
18:3n-3	2.9 ± 0.3	2.6 ± 0.3	— ± —	— ± —	1.5 ± 0.2	1.1 ± 0.1*
18:4n-3	9.2 ± 1.3	8.4 ± 0.1	0.2 ± 0.1	— ± —	4.3 ± 0.8	3.4 ± 0.1
20:4n-3	0.6 ± 0.1	0.7 ± 0.1	— ± —	— ± —	0.4 ± —	0.5 ± 0.1*
20:5n-3	11.0 ± 0.7	17.3 ± 2.2*	14.8 ± 2.3	22.0 ± 2.6*	9.4 ± 0.2	17.5 ± 1.8*
22:5n-3	0.6 ± —	0.7 ± —	— ± —	0.4 ± 0.1*	1.7 ± 0.3	2.4 ± 0.1*
22:6n-3	19.4 ± 0.3	16.3 ± 2.6*	27.1 ± 3.5	34.0 ± 1.1*	35.5 ± 1.8	39.2 ± 2.8*
Sum n-3	44.0 ± 1.2	46.3 ± 4.3	43.2 ± 4.6	57.0 ± 1.7*	52.8 ± 2.4	64.3 ± 2.0*
n-3/n-6	11.0 ± 1.3	10.2 ± 1.4	6.6 ± 2.1	6.2 ± 0.3*	19.6 ± 3.7	23.7 ± 0.8*
Sum 16:1	4.3 ± 0.3	5.6 ± 0.3	2.6 ± 2.2	1.0 ± 0.3*	1.7 ± 0.3	1.6 ± 0.2
Sum 18:1	6.7 ± 0.4	9.2 ± 0.6	1.1 ± 1.0	0.9 ± 1.1	4.8 ± 0.7	4.1 ± 0.2
Sum 20:1	1.6 ± 0.4	1.5 ± 0.1	2.2 ± 0.4	3.2 ± 0.6	0.6 ± 0.2	0.6 ± 0.0
Sum 22:1	— ± —	— ± —	21.9 ± 4.4	21.2 ± 2.6	— ± —	— ± —
Sum mettede	30.2 ± 0.1	24.4 ± 2.2	0.1 ± 0.1	0.3 ± 0.1	29.3 ± 1.0	21.8 ± 1.6*
Sum monoener	13.3 ± 1.0	17.2 ± 1.1	1.8 ± 0.5	1.1 ± 0.3	7.9 ± 1.1	7.8 ± 0.4
Sum n-3	44.0 ± 1.2	46.3 ± 4.3	3.2 ± 0.6	3.6 ± 0.6*	52.8 ± 2.4	64.3 ± 2.0*
Sum n-6	4.0 ± 0.4	4.5 ± 0.2	1.3 ± 2.0	0.1 ± 0.2	2.8 ± 0.4	2.7 ± 0.1
Sum polyener	48.0 ± 0.9	51.4 ± 4.3	— ± —	— ± —*	55.6 ± 2.1	67.0 ± 2.0*

NL, neutral lipids; PE, phosphatidyletanolamine; and PC, phosphatidylcholine. * indicate significant differences between the recovering (3) and half-full (5) stages (Mann-Whitney U-test) sampled 15 September 1997 and 7 February 1998, respectively. Values are given as mean and standard deviation, n = 3.

al. 2002). It is hence likely that the spring bloom in March to April in the present study represented a major input of 20:5n-3 in the scallops with a lack of 22:6n-3, compared with the rest of the year with more abundance of 22:6n-3 in the phytoplankton. The change was probably facilitated by an exchange of gonadal material between March and June, as partial spawnings during spring and early summer were followed by gonad growth (Strohmeier et al. 2000). The major increase in the 20:5n-3/22:6n-3 ratio in the samples from June can hence be attributed to a diet effect. Influence of diatoms in the June samples is supported by the observation of increased ratio of 16:1n-7/16:0 and higher proportion of 16:4n-3, known as indicators of diatoms (Ackman & Tocher 1968, Chuecas & Riley 1969, Napolitano & Ackman 1992), in NL. The ratio and level are not as high, though, as diatom imprints reported by Pedersen et al. (1999) and Napolitano et al. (1997). Similar

changes are reported in other pectinids, with strong increase of 20:5n-3 in various tissues during diatom dominated spring blooms (Pollero et al. 1979, Napolitano et al. 1997). Pollero et al. (1979) found a marked increase in 22:6n-3, particularly in the gonads, associated with the dominance of a dinoflagellate in the stomachs of *Chlamys tehuella*. The importance of diet for the lipid composition of ovaries has also been emphasized in several studies of hatchery conditioning of bivalves (Helm et al. 1991, Utting & Doyou 1992, Soudant et al. 1996a, Soudant et al. 1996b, Soudant et al. 1996c, Soudant et al. 1999).

Changes in the composition of phospholipids due to different temperatures could be expected in the present study, according to mechanisms of temperature adaptation of membrane lipids to maintain a constant or optimal fluidity of the membranes (Williams & Hazel 1994). The temperatures of 8°C to 10°C prior to the

sampling in June and the 8°C to 9°C in December were higher than the winter minima for the March 1997 and February 1998 samples of 5°C to 6°C (Strohmeier et al. 2000). Different strategies for temperature adaptation are described, but usually the degree of unsaturation increases with lower temperatures (Williams & Hazel 1994), which is also seen in bivalves (Ueda 1974, Piretti et al. 1988, Chu & Greaves 1991). In *Placopecten magellanicus*, higher levels of 22:6n-3 in the phospholipids were found in the gonads at lower temperatures and higher depths, whereas higher content of a phytosterol was found in the adductor muscles (Napolitano et al. 1992). These sterols were not measured in the present study. The increased ratio of 20:5n-3/22:6n-3 in PC in June did not give a particular decrease in the degree of unsaturation itself. The fact that the same changes were seen in neutral lipids suggests that these changes were caused mainly by a diet effect as discussed earlier. However, the tendencies to elevated ratio of polyunsaturated to saturated fatty acids in PC in the colder months, relative to both the June and the December samples, may be related to temperature adaptation. This event was probably separate from the shifts in 20:5n-3 and 22:6n-3. In PE, there was an increase in the degree of unsaturation due to the coinciding increases in 20:5n-3 and 22:6n-3 in June (i.e., opposite to a possible effect on unsaturation due to temperature). It seems that the 22:6n-3/20:5n-3 ratio is more regulated in PE compared with PC.

Soudant et al. (1996a, 1996b) reported increased larvae survival under hatchery conditions associated with higher concentrations of 22:6n-3 and a higher 22:6n-3/20:5n-3 ratio in ovary and

egg phospholipids, and similar conditions give better growth in oyster larvae (Thompson & Harrison 1992). Soudant et al. (1996b) further suggested that a 22:6n-3/20:5n-3 ratio in diet greater than 1 was required to avoid deficiency, whereas a ratio greater than 2 is recommended for marine fish (Sargent 1995). Soudant et al. (1996b) also discussed the possibility that elevated levels of 20:5n-3 can have a negative effect on eicosanoid production from 20:4n-6 as seen in fishes (Bell et al. 1994). The differences in 22:6n-3/20:5n-3 ratio that gave impact on egg quality in the study of Soudant et al. (1996c) ranged between 5 and 1 in PC, which was more than the corresponding range from 2.7 to 1 in the present study. The order of magnitude of the changes in the present study may hence not be large enough to affect egg quality. However, the changes probably represent an annual change in the lipid composition status of broodstock from the field. Andersen and Ringvold (2000) found seasonal differences in effect of different broodstock diets on spawning success. This may be related to similar changes in lipid composition status as seen in the present study.

ACKNOWLEDGMENT

The authors thank Kjersti Ask and Thu Thao Nguyen for training and valuable help in the laboratory. The authors also thank Yanic Marty for inspiring methodological discussions. This work was supported by the Norwegian Research Council, project 111388/100 and National Institute of Nutrition and Seafood Research.

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REPRODUCTIVE CYCLE OF COEXISTING MUSSELS, *MYTILUS CALIFORNIANUS* AND *MYTILUS GALLOPROVINCIALIS*, IN BAJA CALIFORNIA, NEW MEXICO

SERGIO CUIEL-RAMÍREZ AND JORGE CÁCERES-MARTÍNEZ*

Centro de Investigación Científica y de Educación Superior de Ensenada, Laboratorio de Biología y Patología de Organismos Acuáticos del Departamento de Acuicultura, Apdo. Postal 2732, 22800, Ensenada, Baja California, México

ABSTRACT The bay mussel, *Mytilus galloprovincialis*, and the California mussel, *Mytilus californianus*, are sympatric species in the Pacific coast of Baja California in northwest Mexico. To determine the reproductive cycle of both species in a coexistence area, a study of quantitative stereology was carried out from January to December 1995. *M. californianus* and *M. galloprovincialis* adults were collected from Mina del Fraile an exposed rocky shore and *M. galloprovincialis* adults were collected in a culture area a protected bay. In both species spawning organisms were found all year round. At population level, *M. galloprovincialis* had one major reproductive season from autumn to early spring in both sites. In late autumn, gonad follicles were full of ripe gametes and developing gametes and some spawning could occur. However, the spawning peak occurred in early winter. Minor spawning conditions were more frequent in *M. galloprovincialis* from the culture area than in *M. galloprovincialis* from the Mina del Fraile. These differences were presumably associated with the most stable environmental conditions in the culture area, which could also influence differences in percentage of storage cells found from spring to summer. The reproductive season of *M. californianus* took place from winter to summer. The major reproductive activity occurred in spring and the resting season took place during autumn. The reproductive season of *M. californianus* in the studied locality is from winter to summer, whereas the reproductive season of *M. galloprovincialis* occurs from autumn to early spring.

KEY WORDS: coexistence, reproductive cycle, spawning, *Mytilus galloprovincialis*, *Mytilus californianus*

INTRODUCTION

The mytilids, *Mytilus californianus* (California mussel) and *Mytilus galloprovincialis* (bay mussel), coexist in the southern part of the west coast of North America. The distribution of *M. californianus* is from Alaska to Islas Socorro in Mexico, and the distribution of *M. galloprovincialis* is from central California to Baja California, Mexico (Soot-Ryen 1955, Mc Donald & Koehn 1988, Koehn 1991, Seed 1992, Suchanek et al. 1997). The genetic divergence between *M. californianus* and *M. galloprovincialis* and their external morphology have been clearly differentiated and, in spite of the fact that they are sympatric species, there is no hybridization between them (Martínez-Lage et al. 1997, Martínez-Lage et al. 2002, Cáceres-Martínez et al. 2003, del Río-Portilla & Cáceres-Martínez, in prep.). Studies on the reproductive cycle of coexisting species aid in explaining their coexistence strategy. Suchanek (1981) from field studies carried out in Alaska and Washington stated that the reproductive strategy of *Mytilus californianus* is based on continuous spawning at a very low level throughout an annual cycle. *Mytilus edulis* (knowing now that bay mussels in Alaska and Washington correspond to *M. trossulus*) is a classic fugitive species that rarely reaches a large size, but matures early and is characterized by a single massive reproductive output each year.

Different techniques have been used to assess the reproductive condition in mussels. A summary on studies of reproduction seasons in mussels from North America is shown in Figures 1 and 2. Seed (1976) from an analysis of reproduction records for several mytilids from various parts of their geographical range noted that mussels from the warmer more southerly waters of the Northern Hemisphere generally spawn earlier than those further north. Southern species usually reproduce later in the year and have a progressively restricted season further north; northern species ex-

hibit the reverse trend, spawning earlier and with a more extended season further south. A wide reproductive period in the southern part of the Northern Hemisphere has also been observed in other studies (Ferrán 1991, Villalba 1995, Cáceres-Martínez & Figueras 1998).

Minor local variations in the reproductive cycle of the same mussels species has also been studied. Seed (1976) described that the gonadal development of *Mytilus edulis* in England was faster in mussels from the low intertidal zone, than in those from the upper zone and he related these results to food availability. Local variations in the gonadal cycle of *Mytilus galloprovincialis* were found in Spain and these differences were associated with environmental conditions (Ferrán 1991, Villalba 1995), but the gonadal cycle of wild *M. galloprovincialis* from the low intertidal exposed rocky shores and from those cultured in floating rafts was similar (Cáceres-Martínez & Figueras 1998).

There are no studies on the reproductive cycle in coexisting *M. galloprovincialis* and *M. californianus* from the same exposed rocky shore in Baja California, Mexico (the southern distribution area of *M. galloprovincialis* and *M. californianus* in the Pacific coast of North America) or on the comparison of the reproductive cycle between *M. galloprovincialis* from exposed and culture conditions. The objectives of this study are to determine the time and duration of the reproductive period of *M. californianus* from exposed conditions, and of *M. galloprovincialis* from exposed and culture conditions through quantitative stereology.

MATERIALS AND METHODS

To determine the reproductive cycle using the proportion of germinal and storage cells in the natural population and the cultured *Mytilus galloprovincialis* and the natural population of *Mytilus californianus*, two samplings were planned from January to December 1995. The first sampling was carried out in an exposed rocky shore named Mina del Fraile south of Bahía de Todos Santos (Fig. 3) where *M. galloprovincialis* and *M. californianus* coexist in

*Corresponding author. E-mail: jcaceres@cicese.mx

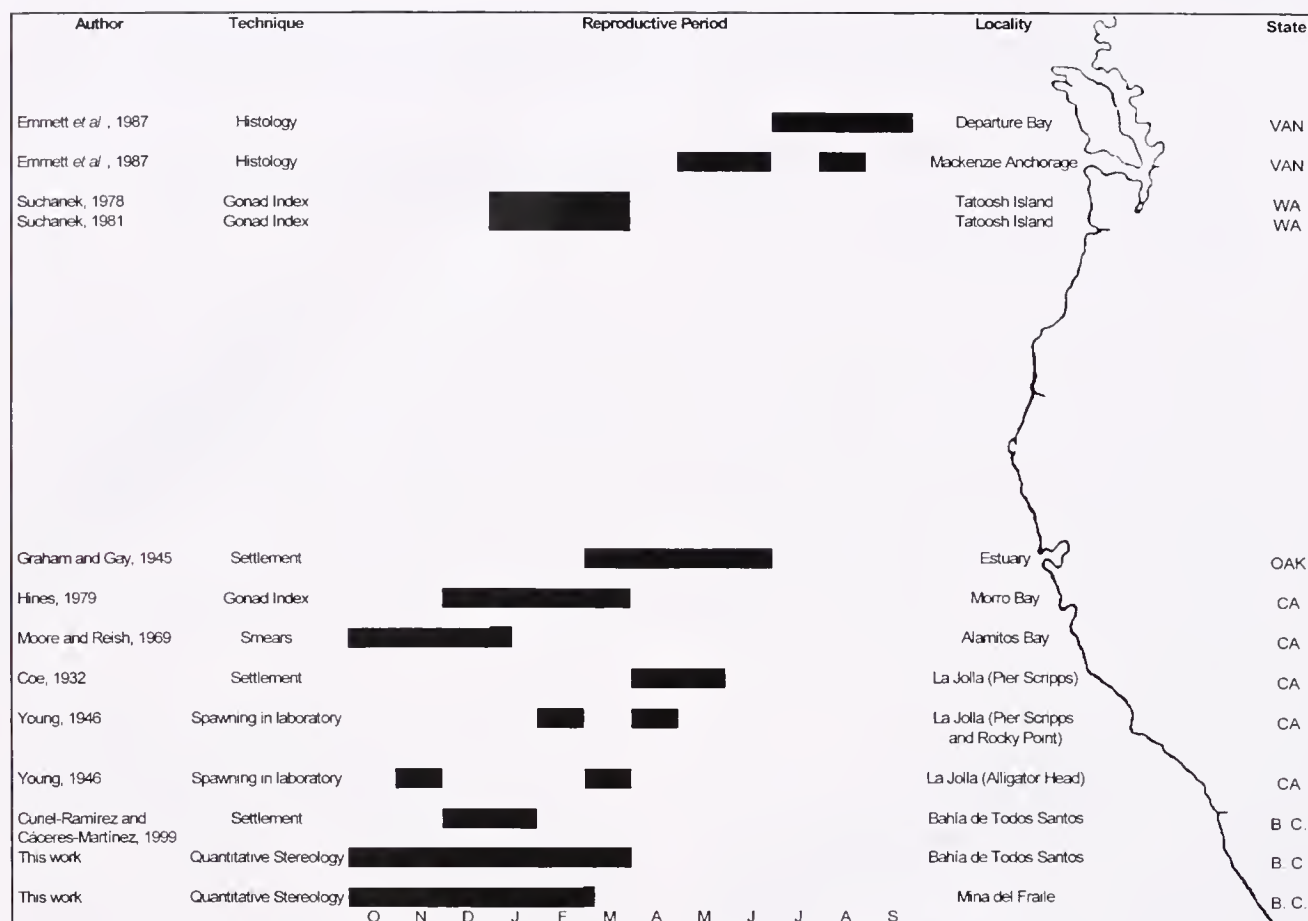


Figure 1. Reproductive cycle of bay mussels along the west coast of North America. All records of *Mytilus edulis*-like from Alaska to northern California were considered as *M. trossulus*, bay mussels from northern California were considered as possible hybrids between *M. trossulus* and *M. galloprovincialis*, while bay mussels from central California to Baja California, México, were considered as *M. galloprovincialis* (Soot Ryen 1955, McDonald & Koehn 1988, Koehn 1991, Seed 1992, Suchanek et al. 1997). The Figure is arranged from north to south.

the intertidal zone. Each month, 30 individual adults from each species were recollected from the middle intertidal zone during low tide periods. The morphologic differentiation of both species at the adult stage was easy. See the morphologic criteria established by Cáceres-Martínez et al. (2003). A total of 360 mussels from each species were sampled; their mean shell length was 57 ± 5.8 mm for *M. californianus*, and 63 ± 8.2 mm for *M. galloprovincialis*. The second sampling was carried out taking 30 *M. galloprovincialis* adults from culture ropes (5 m depth) each month in the culture area located in Bahía de Todos Santos (see Fig. 3). A total of 360 mussels with a mean shell length of 64 ± 6.3 mm were sampled. Mussels from both samplings were brought to the laboratory in plastic bags and were cleaned with a brush and a jet of seawater.

The soft tissue from each mussel was fixed in Davidson's fixative (Shaw & Batle 1957). An anterior transverse section was taken, including the mantle tissue. Histologic sections with a thickness of 5 μ m were cut and stained with hematoxylin and eosin. The presence of gametes and storage cells were determined by quantitative stereology using a Weibel (1969) graticule following the method described by Briarty (1975), Lowe et al. (1982), Seed and Suchanek (1992), and Cáceres-Martínez and Figueras (1998). Five random counts were carried out on each histologic slide. Results are given as a percentage of mantle volume occupied by

the gamete development stages and cell types or mantle components (MC) defined as ripe gametes (RG). In the female, it is the stage in which oocytes were free within the follicles and some oocytes remained attached to the follicle wall; whereas in the male, it is the stage when follicles were filled by spermatozoa arranged in characteristic bands. Similar to the females during the developing stage of gametes (DG) when rounded oocytes along with oocytes were found attached to the follicle wall, in the male varying quantities of developing spermatogenic cells were present. Additional observations on the presence of hemocytes around gametes, and conditions of the follicle walls were recorded to determine if gamete reabsorption was occurring. The other cell components in the gonad were vesicular cells of connective tissue (VCT) and adipogranular cells (AC); empty follicles (EF) were recorded when the points of the graticule indicate an empty area inside the follicle.

The one-way ANOVA was used to determine if gametes (RG and DG) were different among mussel species throughout the study period in both localities and species (Sokal & Rohlf 1987).

RESULTS

Variations in percentage of the different cell types in the mantle volume of *Mytilus galloprovincialis* from the culture area are shown in Figure 4A. RG and DG were found throughout the study

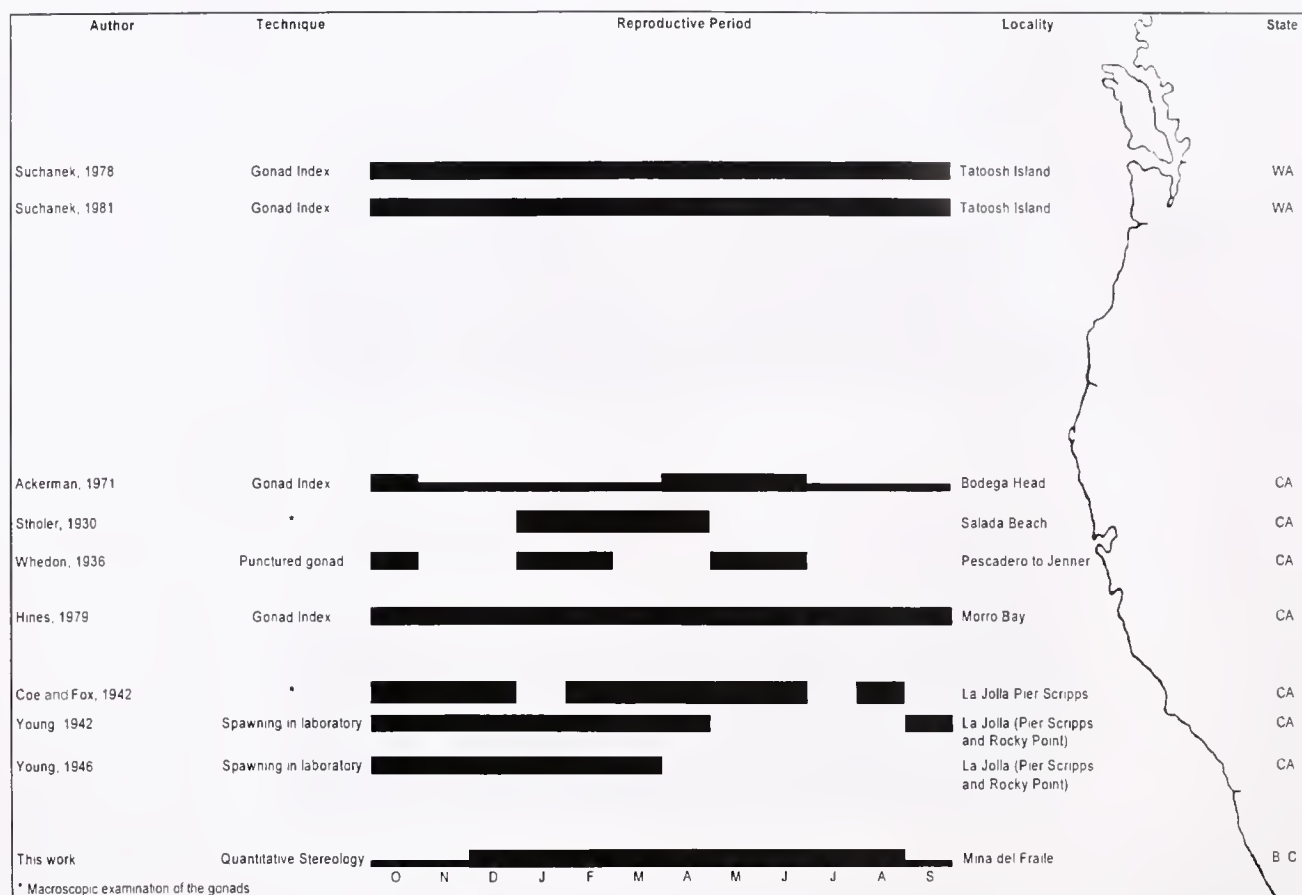


Figure 2. Reproductive cycle of the California mussel *Mytilus californianus* along the west coast of North America. The Figure is arranged from north to south.

period. RG and DG reached their maximum levels in October and November indicating a major spawning period, but remained up to 50% from December to April. EF were from 12.9% in October to 24.2% in March, values of RG and DG in April were around 50%, but gametes were surrounded by hemocytes and follicle walls were in regression, showing a reabsorption period. The reproductive season started in October and ended in March. AC reached their maximum from May to September whereas VCT rose from April to September showing the storage of reserves during spring and summer, which would be used for the next massive gametogenesis. Variations in percentage of the different cell types in the mantle volume of this species from the exposed rocky shore are shown in Figure 4B. RG and DG were also found throughout the study period with their maximum values in November showing a major spawning period, their prevalence remained up to 40% from December to March, when reabsorbing conditions were observed, reaching minimum values from June to September. EF were recorded throughout the study period reaching their higher values from 18.5% in November to 26.6% in February. At this time, AC were in a lower proportion with minimum values in January and February. VCT were present throughout the study period with maximum values from June to September. The percentage of MC was similar to those described above for this species in culture conditions from November to February; and it was different from March to September, the period when the accumulation of reserves was more intense. In general, differences in percentage of gametes

throughout the study period in both localities were significant ($F_{(2, 4907)} = 301, P = < 0.01$).

The variations in percentage of the different cell types in the mantle volume of *Mytilus californianus* from the exposed rocky shore are shown in Figure 4C. RG and DG were found throughout the study period with minimum values from August to December, while maximum values were recorded from January to July (up to 40%). Their maximum values were reached from February to June showing the major spawning activity. EF were recorded throughout the study period at similar percentage values. AC were recorded in all the months sampled, with maximum values in September and October (storage of reserves); their minimum values were reached from March to May. VCT were present throughout the study period with maximum values from September to December. Differences in percentage of gametes throughout the study period in both localities and species were significant ($F_{(2, 4907)} = 301, P = < 0.01$).

DISCUSSION

The reproductive season of *Mytilus galloprovincialis* cultured in Bahía de Todos Santos is more extended than those from the same species in the exposed rocky shore from Mina del Fraile. These observations could be related to environmental conditions. Culture mussels feed underwater at all times and they are not exposed to environmental changes such as desiccation or to sharp

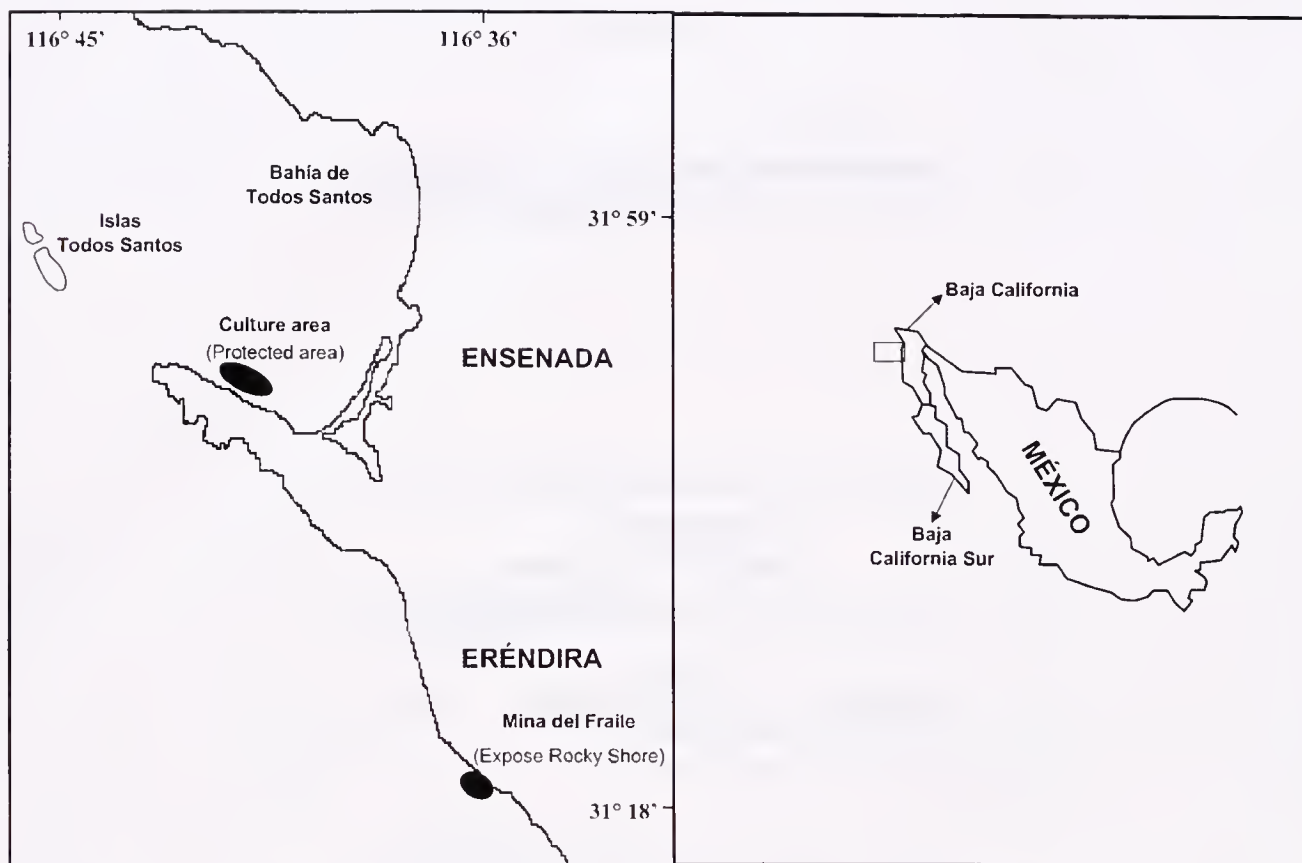


Figure 3. Map showing the mussel culture area in Bahía de Todos Santos and the exposed rocky shore in la Mina del Fraile, Baja California; where *Mytilus galloprovincialis* and *Mytilus californianus* were collected for the study.

temperature changes due to tides at midnight or midday. Thus, external conditions during the season of reserves accumulation (i.e. temperature changes, food availability, wave splash, and photoperiod) seem to be more stable in the culture condition than in exposed rocky shore conditions, allowing a more frequent and intense reproduction (see Fig. 4, A,B). Seed (1976) described that the gonadal development was faster in mussels from the low intertidal than in those from the upper intertidal, relating these results to food availability. Ferrán (1991) and Villalba (1995) associated local variations in the gonadal cycle of mussels cultured in Galician bays in Spain to environmental conditions.

Comparing the reproductive season of *M. galloprovincialis* with *M. californianus* it is observed that in some months the percentage of MC was similar in culture or exposed rocky shore conditions; however, the reserves accumulation period between species shows differences in reproductive cycles. The most intense spawning period in *M. californianus* occurred from middle winter to spring. It is clear that *M. californianus* presents a more extended reproductive season from winter to summer, suggesting that endogenous factors, regulating the reproductive cycle in this species, and the exogenous factors act in conjunction in different ways in comparison to *M. galloprovincialis*.

A comparison of the reproductive season of *M. galloprovincialis* found in this study at regional scale showed a similar pattern to those described for the bay mussel from Washington to southern California (see Fig. 1), which shows a reproductive season between October and April, with the exception of the studies by Graham and Gay (1945), Coe (1932) and Young (1946) in the Pier of Scripps Institution of Oceanography and Rocky Point. The first

two authors arrived to the conclusion that through spatfall studies; thus it is probable that spawning period could be earlier than stipulated. The last author came to his conclusion from spawning induction in laboratory, which does not represent the reproductive conditions of the mussel population in the wild. There seems to be a pattern of reproduction period from autumn to early spring of bay mussels from Washington to Baja California and from spring to summer in Vancouver Island. Are these differences related to the bay mussel species *Mytilus trossulus* towards north, and *Mytilus galloprovincialis* towards south? Or to latitudinal and local conditions? A comparison on reproductive seasons of the bay mussels (*M. trossulus* and *M. galloprovincialis*) is necessary using the same technique in the north western coast of North America to determine the reproductive strategy of these two bay mussel species.

On the other hand, reproductive periods of *Mytilus californianus* recorded in the Pacific coast of North America (see Fig. 2) show spawning periods almost all year round. Our results and those from the mentioned studies in Figure 2, support in part the observations by Suchanek (1981) mentioning that the reproductive strategy of *M. californianus* is based on continuous spawning at very low level throughout an annual cycle. However, more studies on the spawning intensity or level of this species all year round in comparison to *M. galloprovincialis* are needed to confirm the observation by Suchanek.

The results of this study show that *M. galloprovincialis* has a less extended reproductive season in the rocky shore exposed environment than in the culture conditions, and the reproductive cycle of this species in both localities is less extended than those

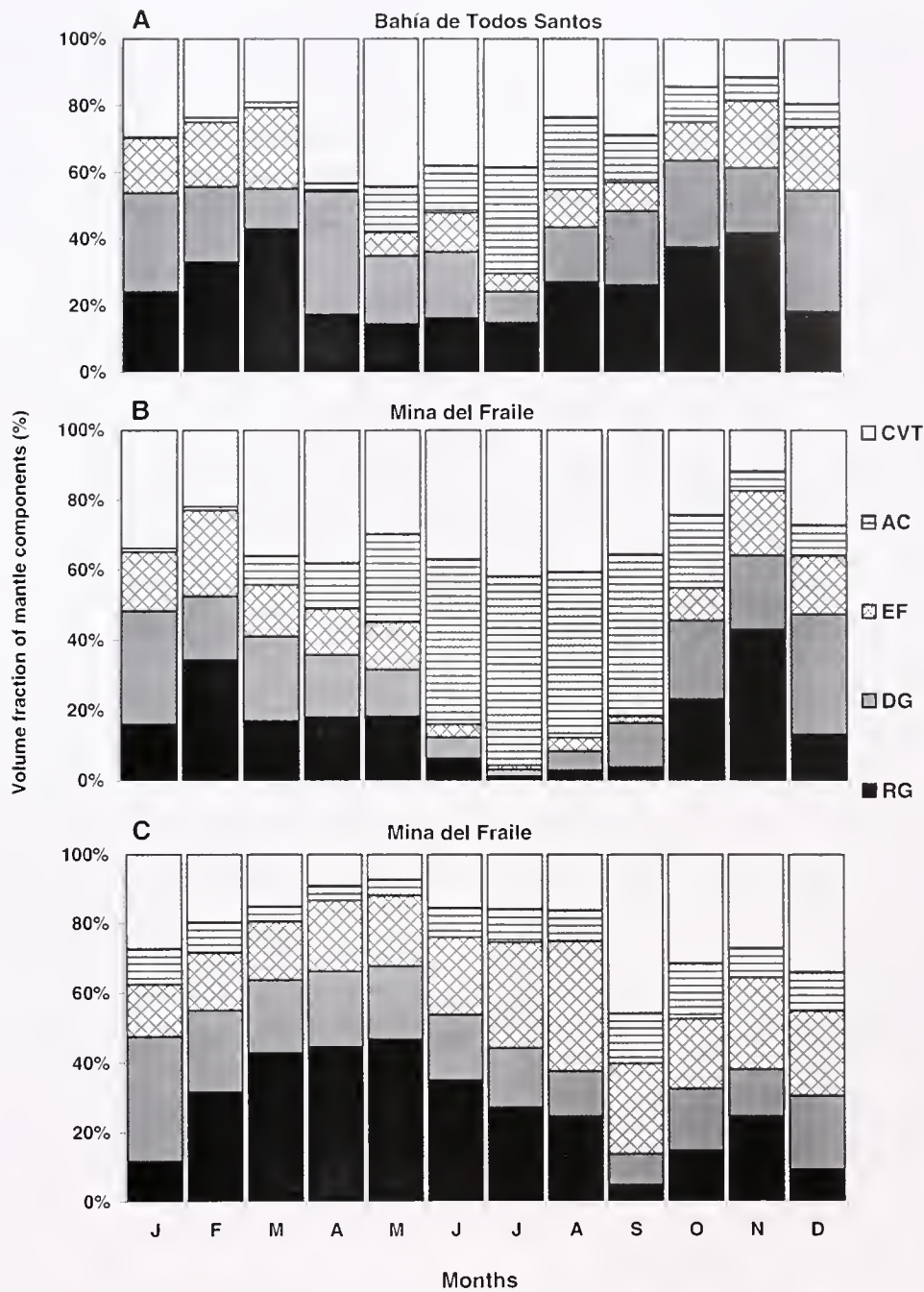


Figure 4. Variations of the volume percentage of mantle components of *Mytilus galloprovincialis* from the culture area (A), *Mytilus galloprovincialis* from the exposed rocky shore (B), and *Mytilus californianus* from the exposed rocky shore (C), during the study period. Ripe gametes (RG), Developing gametes (DG), empty follicles (EF), adipogranular cells (AC) and vesicular cells of connective tissue (VCT).

of *M. californianus* in the exposed rocky shore environment. This period takes place from autumn to early spring, whereas for *M. californianus* it takes place from winter to summer, both species having smaller spawning periods all year long.

The mussel settlement season in Bahía de Todos Santos has been recorded in December and January (Curiel-Ramírez & Cáceres-Martínez 1999, Curiel-Ramírez 2000), coinciding with the spawning observed for *M. galloprovincialis* in this study. Current studies on the identification of recently settled mussels supports the fact that the majority of the spat from these months correspond to this species (Curiel-Ramírez & Cáceres-Martínez, in

prep), such as it had been mentioned about the necessity of studies on the intensity of spawning in these species. Also, studies are necessary on the settlement intensity of both species by identification of recently settled mussels during the year.

ACKNOWLEDGMENTS

The authors thank S. Guevara from Acuacultura Oceánica for allowing us technical assistance for sampling in their mussel culture facility. The authors also thank Rebeca Vasquez for processing samples. This project was supported by the CICESE project number 623106. Curiel-Ramírez received a grant from Conacyt, Mexico.

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TEMPORAL VARIATION IN THE SETTLEMENT OF BLUE MUSSELS (*MYTILUS EDULIS* C. LINNAEUS, 1758 AND *M. TROSSULUS* GOULD 1850) IN EASTERN MAINE

PAUL D. RAWSON,* AFTON MCGOWEN, AND CHRIS BARTLETT

School of Marine Sciences University of Maine, Orono, Maine, 04469 and Maine Sea Grant Program Eastport, Maine 04631

ABSTRACT Blue mussels in the genus *Mytilus* are cultured extensively in the Northeastern United States and throughout much of Atlantic Canada. Recent reports suggest that of the two species that co-occur in this region, the economic value of *Mytilus edulis* is higher than that of its congener *M. trossulus*. Because mussel farmers typically rely on wild caught spat to seed grow-out facilities, the productivity of mussel farms may benefit from a method of spat collection that limits the frequency of *M. trossulus* spat. To explore the potential for developing such a method in eastern Maine, where *M. trossulus* is highly abundant, we investigated the spatial and temporal variation in the proportion of *M. edulis* and *M. trossulus* spat settling at three sites in Cobscook Bay, Maine, USA in 2000. We found little evidence of location- or depth-dependent variation in the frequency of *M. edulis* spat. There was a significantly greater proportion of *M. edulis* spat on the collectors retrieved in October and December relative to those retrieved in August and September. However, the low abundance of settlers in October and December, coupled with the likelihood that spat settling late in the year will experience low growth rates and high mortality as a function of rapidly declining water temperature and food supplies, suggests that there are significant impediments to the preferential collection of *M. edulis* seed within Cobscook Bay.

KEY WORDS: settlement, spat collection, mussel culture, spatial variation, temporal variation, *Mytilus*

INTRODUCTION

The blue mussel, *Mytilus edulis* (C. Linnaeus 1758), is a common, rocky intertidal and subtidal bivalve mollusk, which is harvested extensively in the Northeastern United States and Atlantic provinces of Canada. Despite a growing market demand for blue mussels in the U.S., the domestic production of mussels has steadily declined from a peak of 4800 metric tons in 1988 to 1400 metric tons in 2001 (NMFS 2003). Among the states contributing to the U.S. production, the vast majority (~88%) are harvested in Maine. Historically mussels have been harvested from natural beds and bottom culture leases seeded with juvenile mussels. Recently, however, mussel culturists in Maine have adopted suspended raft techniques commonly used in parts of Europe and Atlantic Canada. Because off-bottom techniques use a much greater proportion of the water column they are much more efficient and produce a product considered to have superior meat yield and quality relative to that obtained from bottom culture (Lutz 1980, Hickman 1992, Roberts et al. 1999). Although the majority of suspended raft and other off-bottom culture operations are presently located on the central coast of Maine, potential suspended raft culture sites can be found throughout much of the state.

The presence of a second species of blue mussel, *M. trossulus* (Gould 1850), however, may present an impediment to the expansion of raft culture to sites in eastern Maine. Genetic surveys using allozyme electrophoresis and PCR-based molecular markers have shown that *M. trossulus* is widely distributed throughout the Canadian Maritime Provinces, often in mixed populations with *M. edulis* (e.g., Koehn et al. 1976, 1984, Bates & Innes 1995, Comesaña et al. 1999, Innes & Bates 1999, Mallet & Carver 1999, Penney & Hart 1999, Penney et al. 2002). Recently, Rawson et al. (2001) found that blue mussel populations throughout much of eastern Maine contain relatively high frequencies of *M. trossulus*, as well. Freeman et al. (1994) have shown that *M. trossulus* mus-

sels obtain less meat than *M. edulis* mussels and are more prone to shell fracture during processing. In addition, given species-specific growth rates, Mallet and Carver (1995) have estimated that the economic value of *M. edulis* is 1.7 times that of *M. trossulus* under off-bottom culture conditions. Because raft culture facilities are typically seeded with wild caught mussel spat, the proportion of *M. edulis* and *M. trossulus* under cultivation is dependent on the relative frequency with which these species settle on spat collectors. Thus, in locations where *M. edulis* and *M. trossulus* are sympatric, a method of spat collection that favors *M. edulis* seed and limits the frequency of *M. trossulus* seed would be highly beneficial to the productivity of suspended culture facilities (Freeman 1996).

The development of such methods, in turn, is dependent upon there being sufficient temporal or spatial variation in species-specific settlement. Freeman et al. (2002) and Kenchington et al. (2002) explored the potential for the differential settlement of *M. edulis* and *M. trossulus* larvae at depth in mesocosm and field studies, respectively. The results of their mesocosm study suggest that species-specific larval behaviors result in the greater settlement of *M. edulis* at depth when there is no thermocline present. In their field studies, they examined the patterns of mussel settlement at two sites in Nova Scotia, Canada, throughout the spawning season in 3 successive years. Although the timing of species-specific settlement varied among years, they consistently observed a significantly higher percentage of *M. edulis* spat on collectors deployed 4–5 m below the surface. Based on their results, they recommended that mussel growers consider deploying subsurface spat collectors to increase the proportion of *M. edulis* postlarvae.

In this study, we investigated the spatial and temporal variation in the frequency of *M. edulis* and *M. trossulus* settlement at three sites in Cobscook Bay, Maine, USA to determine whether such variation will facilitate the development of spat collection methods favoring *M. edulis* and the expansion of raft culture operations to eastern Maine. Although our sampling strategy differs somewhat from that of Kenchington et al. (2002), we were particularly interested in whether the increased settlement of *M. edulis* with depth observed by Kenchington et al. (2002) is found elsewhere in the zone of sympatry between *M. edulis* and *M. trossulus*.

*Corresponding author. E-mail: prawson@maine.edu

MATERIALS AND METHODS

Spat Collection

The temporal and spatial patterns of mussel settlement were investigated by placing spat collectors at three locations in Cobscook Bay (Fig. 1). Cobscook Bay is an extremely dynamic embayment where the morphology of the bay in combination with a large tidal range (>8 m) creates strong directional flows within much of the bay (Brooks et al. 1999). We placed spat collectors at three sites that lie within the major flow patterns within the bay. All three sites were located 30–50 m from shore in approximately 35–40 m of water (at low tide). Collectors were suspended from the peripheral anchor lines and buoys of salmon net pens at Shackford Head (SH), from an equipment supply raft on a salmon aquaculture site in South Bay (SB), and from the anchor lines of an experimental scallop farm in East Bay (EB). Spat collectors consisted of 6 m lengths of aged and frayed 13-mm thick polypropylene rope, also known as “pot warp”. Each collector was suspended from the surface and weighted at the bottom using cinder blocks (12.3 kg) to keep the collector vertical in the water column. Three replicate collectors were initially deployed at each site on June 27, 2000 and recovered on August 8 at which time a second set of collectors was deployed. Collectors were recovered and redeployed on September 28 and October 28, and the experiment was terminated when the last set was retrieved on December 28. Upon recovery, the spat collectors were cut into three 1.5-m sections (top, middle, and bottom) starting from the surface. Each section was placed in an individual Ziploc bag containing 95% ethanol, and transported on ice to the University of Maine where they were stored at 4°C. Subsequently, each rope section was removed from the 95% ethanol, the rope strands were unwound, and all spat gently removed. These spat were combined with any

that had fallen off and settled to the bottom of the Ziploc bag, counted by hand, placed in 50-ml centrifuge tubes containing fresh 95% ethanol, and stored at 4°C.

Molecular Analysis

Previous studies investigating genetic variation in northwest Atlantic blue mussel populations have used various combinations of allozyme and PCR-based markers to identify *M. edulis* and *M. trossulus* individuals and their hybrids (e.g., Koehn et al. 1976, 1984, McDonald et al. 1991, Bates & Innes 1995, Comesaña et al. 1999). Because PCR-based methods require very little tissue they are ideally suited to studies involving larvae or recently settled postlarvae. Thus, we estimated the proportion of *M. edulis* and *M. trossulus* among the spat on each section of rope using two PCR-based genetic assays. Template DNA was prepared from approximately 20 individual spat from each rope section by placing whole spat in a 0.5-mL microcentrifuge tube containing 20 ng of proteinase K in 10 µL of a buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100 and incubating at 55°C for 3 h on a rotating platform. The samples were heated to 95°C for 15 min before 10 µL of GeneReleaser (BioVentures Corp.) was added. The resulting mixtures were incubated at 65°C for 30 s, 8°C for 30 s, 65°C for 90 s, 97°C for 180 s, 8°C for 60 s, 65°C for 180 s, 97°C for 60 s, and finally 65°C for 60 s, after which the samples were centrifuged at full speed (13,200 rpm) for 2 min. The supernatant containing DNA was transferred to a new 0.5-mL microcentrifuge tube and was stored at 4°C. The isolated DNA was used in the PCR-based Glu-5' and ITS genetic assays that are considered diagnostic for *M. edulis* and *M. trossulus*. The application of these markers followed the protocols described in Rawson et al. (1996) and Heath et al. (1995), respectively.

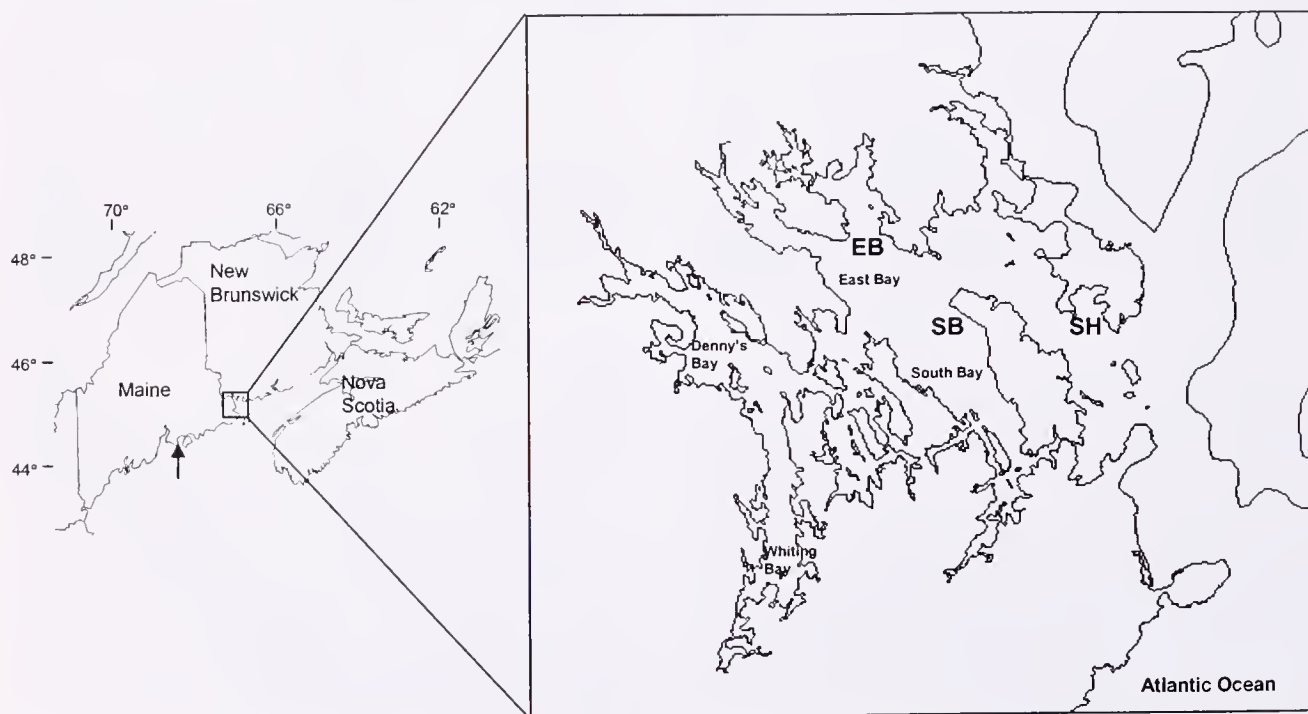


Figure 1. Locations in Cobscook Bay, Maine, USA where spat collectors were deployed. Site designations are given in the text. The arrow in the left panel indicates the approximate location of the Blue Hill Salt Pond where, at present, the majority of mussel seed are obtained by mussel growers in Maine.

Statistical Analysis

Variation in the combined settlement of *M. edulis* and *M. trossulus* with respect to site, date of collection, and depth was analyzed by a 3-way full factorial fixed effects (model I) analysis of variance (ANOVA). Of the 36 ropes deployed in this study, four were lost prior to recovery. Although no more than one rope was lost at any single location during any sampling period, this creates imbalance in our dataset so that the appropriate F statistics for the main effects and all interaction terms were estimated using Type III sums of squares. To eliminate heteroscedasticity among the residuals, total counts of spat abundance were log transformed prior to analysis. The validity of the model and data transformation was further determined by testing whether the residuals were normally distributed using a Levene's test.

Variation in the frequency of *M. edulis* Glu5' and ITS alleles among the spat recovered from individual rope segments was also analyzed using ANOVA. For many of the ropes retrieved in August, October, and December, we were unable to find and genetically characterize more than a couple (<5) spat per rope segment (Table 1). This created a severe imbalance in our dataset and precluded the use of a 3-way ANOVA to simultaneously analyze site- and depth-specific variation in the frequency of *M. edulis* alleles across all four sampling periods. Thus, to analyze temporal variation in the abundance of *M. edulis* spat, we first calculated the *M. edulis* Glu5' allele frequency among the spat collected and genotyped at each site within each month, without regard for individual ropes or depths. The site-specific allele frequencies were then analyzed in a 1-way ANOVA with sampling date as the independent variable. One of our main goals in this study is to investigate whether species-specific settlement varies as a function of depth. In this regard, we obtained sufficient allele frequency data to analyze the depth- and site-specific variation in the frequency of the *M. edulis* Glu5' allele for the ropes collected in September using a 2-way fixed effects ANOVA. In both analyses, the frequency of the *M. edulis* Glu5' allele was normalized using an arcsin-transformation. The appropriate F-statistics for the main effects and any interaction terms were constructed using Type III sums of squares and the validity of the model and data transformation determined by graphical analysis of the homogeneity of the residuals and testing whether the residuals were normally distributed using a Levene's test. An identical approach was also used to examine the degree to which variation in the frequency of the *M. edulis* ITS allele was related to location, depth, or sampling date.

RESULTS

We observed substantial spatial and temporal variation in the settlement of blue mussels, *M. edulis* and *M. trossulus*, in Cobs-

TABLE 1.

Number of rope segments (spat collectors) for which ≥ 10 spat were retrieved and genetically identified during the 4 sampling periods in this study. Three depths were sampled with T, M, and B corresponding to the top, middle, and bottom 1.5 m rope segments, respectively.

	August			September			October			December		
	T	M	B	T	M	B	T	M	B	T	M	B
Shackford Head	0	2	0	3	3	3	2	2	2	1	2	2
South Bay	0	0	0	3	2	3	1	1	1	0	2	1
East Bay	1	1	2	2	2	1	1	1	1	0	0	0

cook Bay. The total abundance of mussel spat on rope segments varied from 0 on collectors retrieved from East Bay in December to nearly 1,500 per 1.5 m rope segment at Shackford Head in September (Fig. 2). The 3-way ANOVA model explained over 90% of the variance in settlement in our data set (adjusted $R^2 = 0.90$; $F = 15.06$; $P < 0.001$) and identified a significant interaction involving all three of the main effects in the model (Table 2). This interaction is primarily due to a shift from a positive association between the abundance of settlers and depth in August and December to a negative association in September and October at Shackford Head, and only a weak dependence of spat abundance on depth at the other two sites (see Fig. 2). Two of the main effects in this model, month and site, were also significant. In general, blue mussel settlement was much higher at Shackford Head compared with both the East Bay and South Bay sites, up to 5-fold higher in the September and October collections. Peak settlement occurred in September at all three locations, whereas much lower settlement was observed on the collectors retrieved in October and December and virtually no spat were found on the collectors recovered in August.

We isolated DNA from a total of 1316 spat sampled from 48 rope segments and successfully genotyped 1249 (95%) of these individuals at the Glu5' marker. Our success rate for the ITS marker was lower and only 1022 spat were successfully genotyped at this marker. Of the 1020 individuals that successfully genotyped both markers, the allele frequency variation at the Glu5' marker was highly correlated with the variation at the ITS marker across sampling date, site, and depth ($r = 0.95$; $P < 0.001$). Because the statistical analyses of the variation at the Glu5' and ITS markers are quantitatively and qualitatively similar and the higher success rate for the Glu5' marker, we have chosen to present only the ANOVA results pertaining to the spatial and temporal variation in the frequency of the *M. edulis* Glu5' allele.

The frequency of the *M. edulis* Glu5' allele was fairly consistent within collection dates and across depths and the majority of the variation in *M. edulis* Glu5' allele frequency occurred between sampling dates (Fig. 3). Unfortunately, the lack of settlers on many of our collectors precluded a formal statistical analysis of allele frequency variation with respect to depth at all three sites across all four collection dates. However, we pooled the frequency data across sites and depths and used a 1-way ANOVA to investigate whether there was significant temporal variation in the frequency of the *M. edulis* Glu5' allele. This model was highly significant (adjusted $R^2 = 0.95$; $F = 41.00$; $P < 0.001$; Table 3) and *post hoc* tests, (Tukey's) of means indicated that significant differences in the average allele frequency of the *M. edulis* Glu5' allele occurred between sampling dates. This significant difference is due to an increase in the frequency of the *M. edulis* Glu5' allele from approximately 40% on ropes retrieved in August and September to 80% on the ropes retrieved in October and December (Fig. 3 & 4). In addition, we obtained genotypic data from enough of our collectors in September to allow us to use a 2-way ANOVA to investigate the degree to which the frequency of the *M. edulis* Glu5' allele varies as a function of sampling location and depth. This ANOVA model explained only 43% of the variation in the arcsin-transformed allele frequency for the collectors retrieved from all three sites in September (adjusted $R^2 = 0.43$; $F = 1.21$; $P = 0.37$; Table 4). The low R^2 value indicates that most of the variation in *M. edulis* Glu5' allele frequency occurred among individual rope segments. Not surprisingly, neither the 2-way interaction nor the main effects were significant in this analysis, although it should be

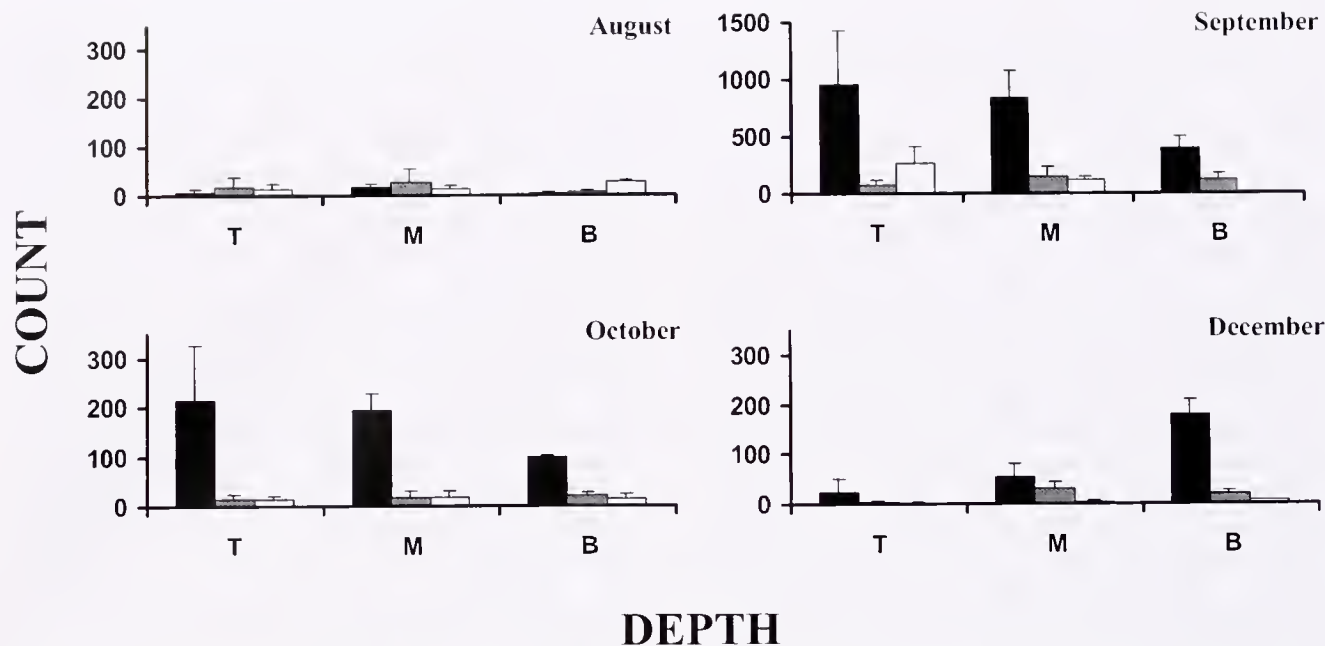


Figure 2. The mean abundance (count) of blue mussel spat (*Mytilus edulis* and *M. trossulus*) on rope collectors at Shackford Head (solid bars), South Bay (grey bars), and East Bay (white bars) as a function of sampling date and depth. Rope collectors were divided into 3 segments, one from 0–1.5 m below the surface (T), a second 1.5–3 m below the surface (M), and the third 3–4.5 m below the surface (B). Error bars represent the mean +1 standard deviation.

noted that this ANOVA had relatively little power to detect such effects (see Table 3).

We used the information from both of our genetic markers to estimate the frequency of individuals with *M. edulis*, *M. trossulus*, and mixed di-locus genotypes. Boecklen and Howard (1997) have shown that at least 4–5 well-differentiated genetic markers are necessary to reasonably estimate the frequency of backcross hybrid genotypes. We successfully genotyped 1200 mussel spat at two diagnostic markers. Though this is too few markers for estimating introgressed genotypes, we can use our genotypic data to roughly classify individuals into *M. edulis*, *M. trossulus* and mixed or hybrid genotypes. The frequency of *M. trossulus* and hybrid genotypes both peaked in September and then declined to around 12% and 4%, respectively, in our October and December samplings (Fig. 5). As expected from the shift in the frequency of the *M. edulis* Glu5' allele frequency, *M. edulis* genotypes were predominant on the collectors retrieved in October and December.

TABLE 2.

Three-way ANOVA (full model) of log-transformed settlement frequency for blue mussel spat at 3 sites in Cobscook Bay during 2000.

Source	df	SS	F _{ratio}	P
Month	3	105.82	85.44	0.000
Site	2	42.35	51.29	0.000
Depth	2	2.75	3.31	0.053
Month * Site	6	35.61	14.38	0.000
Month * Depth	6	14.02	5.66	0.000
Site * Depth	4	2.19	1.32	0.272
Month * Site * Depth	12	10.66	2.15	0.027
Residual	58	23.95		

Finally, we used the genotype frequency data to estimate the abundance of *M. edulis* spat at the Shackford Head site as a function of sampling date and depth. The highest abundance of settlers was observed at Shackford Head; however, given the temporal shift in genotype frequencies, the abundance of *M. edulis* settlers remains relatively constant through time (Table 5)

DISCUSSION

The mussel culture industry in Maine currently uses wild caught spat to seed both bottom and off-bottom culture sites. In central Maine, the majority of mussel seed for off-bottom culture originate from the Blue Hill Salt Pond (see Fig. 1). The expense and logistics of seed transfer between central and eastern Maine, as well as concern regarding the potential for disease transfer associated the movement of shellfish between rivers and bays in Maine make it highly desirable to identify a source of mussel seed in eastern Maine. The studies of Freeman (1994) and Mallet and Carver (1995, 1999), however, indicate that the productivity of off-bottom culture operations can be substantially decreased when there is an increased frequency of *M. trossulus* under culture. Given the prevalence of *M. trossulus* adults in Cobscook Bay and well into the Gulf of Maine (Rawson et al. 2001), capitalizing on local sources of seed requires the development of spat collection methods that limit the frequency of *M. trossulus*. For this reason, we investigated species-specific patterns of settlement among the mussel spat settling on our collectors in Cobscook Bay.

Larval settlement in marine benthic invertebrates, particularly those with an extended period of planktonic development, is the result of an interplay between various factors including local and regional hydrodynamics (Pineda 2000, Sponaugle et al. 2002). The morphology of Cobscook Bay together with the large tidal range in the region (>8 m) creates strong directional flows in the main channel of the bay (Brooks et al. 1999). As these tidal flows enter

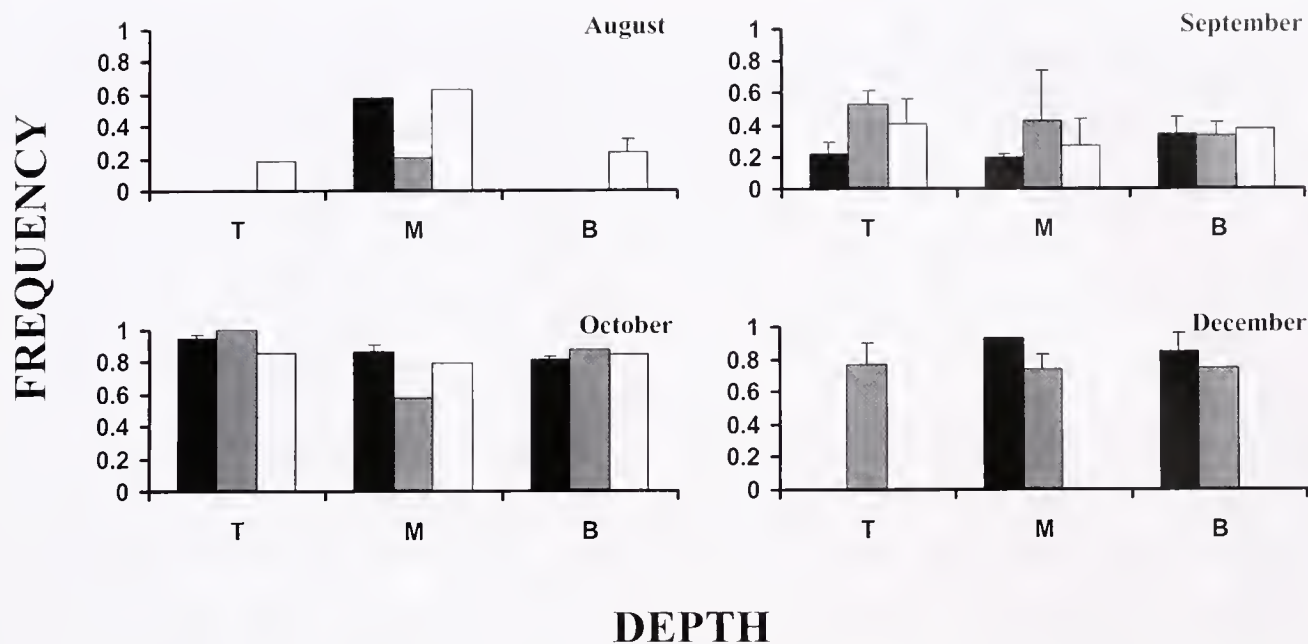


Figure 3. The average frequency of the *M. edulis* Glu5' allele among spat settling at each of 3 collecting sites as a function of sampling date and depth. Depth and location are the same as in Figure 2. Error bars represent the mean ± 1 standard deviation, which were estimated from arcsin-transformed values and then back-transformed for clarity.

the center of the bay, they in turn create an eddy dipole consisting of one clockwise gyre in the southern portion of East Bay and a counter-clockwise gyre in the northern portion of South Bay (Brooks et al. 1999).

Our collectors were deployed near these hydrodynamic features and we anticipated large numbers of mussel postlarvae at all three of our sites. However, we observed substantial variation in mussel settlement among sites. The relatively low settlement on the collectors at our South Bay site was surprising, given that these collectors were placed at a salmon net pen facility within the main current associated with the South Bay gyre. Heavy fouling of the net pens by blue mussels is a continuing problem at this facility (C. Bartlett, personal observation). We can only surmise that because our collectors were downstream of the net pens they were essentially in a settlement shadow. Likewise, the low settlement at our East Bay site suggests a diminished supply of postlarvae. In this case, we feel that our collectors were located further north of the East Bay gyre than we had initially intended.

In contrast, we observed much higher settlement at our Shackford Head site. The value of sites like Shackford Head as a local source of mussel spat within Cobscook Bay, however, is dependent upon the degree to which settlement for *M. edulis* and *M. trossulus* is temporally or spatially segregated. We found no evidence of location-specific variation in the relative abundance of *M. edulis* and *M. trossulus* spat on our collectors. This observation is

consistent with our previous observation that *M. trossulus* adults are highly abundant throughout Cobscook Bay (Rawson et al. 2001). Blue mussels have a planktonic development period that typically lasts 30 days or more and likely results in an open population structure providing little opportunity for spatial variation in the abundance of *M. trossulus* or *M. edulis* larvae to develop.

We found little evidence of depth-specific variation in the abundance of *M. edulis* spat, as well. Low settlement on many of our collectors prevented us from analyzing the effect of depth on

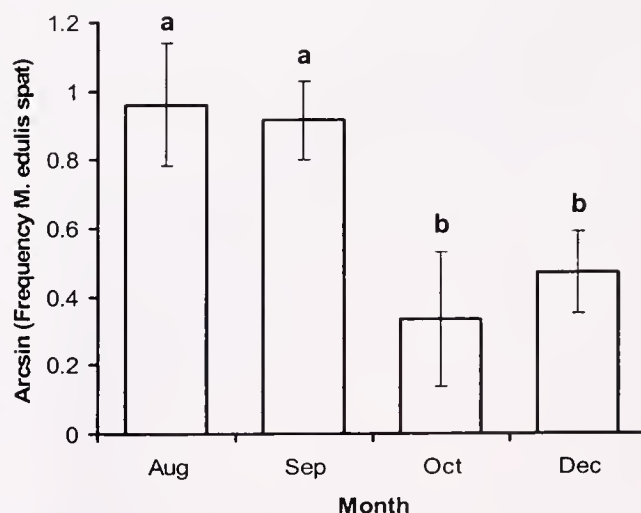


Figure 4. The average frequency of the *M. edulis* Glu5' allele among blue mussel spat settling at all 3 collecting sites and depths combined, as a function of sampling date. Error bars represent the ± 1 standard deviation for the arcsin-transformed values. The lowercase letters above each bar indicate sampling dates for which a *post hoc* test of means (Tukey's) indicated there were significant differences in the mean frequency of the the *M. edulis* Glu5' allele.

TABLE 3.

One-way ANOVA of arcsine-transformed *M. edulis* Glu5' allele frequency at 3 sites in Cobscook Bay across sampling dates in 2000.

Source	df	SS	F _{ratio}	P
Month	3	0.655	41.00	0.0002
Residual	6	0.032		

TABLE 4.

Two-way ANOVA of arcsine-transformed *M. edulis* Glu5' allele frequency at 3 sites in Cobscook Bay in September, 2000.

Source	df	SS	F _{ratio}	P	Observed power*
Site	2	0.13	2.66	0.108	0.44
Depth	2	0.01	0.25	0.785	0.08
Site * Depth	4	0.09	0.91	0.488	0.21
Residual	13	0.31			

* α set to 0.05.

the frequency of *M. edulis* markers at all sampling dates. However, depth had no effect on either the frequency of the Glu5' nor the ITS *M. edulis* allele in September when spat abundance on our collectors peaked. The maximum difference between the average depth-specific frequency of the *M. edulis* Glu5' allele was 0.06%. Although the ANOVA testing for depth-specific variation had low statistical power, *post hoc* analysis suggests that even if we had deployed five times as many collectors as we did in this study, the power to detect such small differences would still have been <0.3.

The lack of depth-specific variation in the abundance of *M. edulis* spat was unanticipated, given the recent report by Kenchington et al. (2002). They examined the species-specific patterns of blue mussel settlement at two sites in Nova Scotia and observed consistently higher settlement of *M. edulis* on collectors 4–5 m below the surface. The discrepancy between our study and that of Kenchington et al. (2002) may be due to the different sampling strategies used in each. Kenchington et al. (2002) genotyped mussel spat from collector segments that were 0–1 m, 2–3 m, and 4–5 m below the surface. Thus, our sampling strategy did not extend as deep (only 4.5 m) and our sampling intervals were not as discrete as those of Kenchington et al. (2002). It is interesting to note, however, that Kenchington et al. (2002) observed an increase in the frequency of *M. edulis* spat even at 2–3 m below the surface at their Parrang Cove site (see Fig. 3 in Kenchington et al. 2002). A similar increase in the frequency of *M. edulis* postlarvae below 3 m in Cobscook Bay should have been detected by our methods.

A significant shift in the frequency of *M. edulis* Glu5' and ITS alleles was apparent across sampling dates in our study. The average frequency of these alleles increased from 40% to 45% in August and September to 75% to 80% in October and December. This shift was unexpected, given the overlapping reproductive cycles for *M. edulis* and *M. trossulus* in Cobscook Bay. Maloy et al. (2003) examined the seasonal changes in gamete volume fraction and oocyte diameter for an intertidal population of blue mussels located near our East Bay collectors. They found that maximal gonadal development for both species occurred in mid July followed by a spawning event in late July and early August of 2000. Assuming a 30-d period of larval development, the timing of the spawning event observed by Maloy et al. (2003) correlates well with the peak settlement we observed at our three sites in September. Similarly, Toro et al. (2002) observed overlapping reproductive cycles for *M. edulis* and *M. trossulus* in a sympatric population in Newfoundland. They also noted a protracted period of spawning activity for *M. trossulus* and hybrid mussels. The increase in the frequency of *M. edulis* spat in October and December in our study, however, would suggest a more protracted spawning season for *M. edulis* adults in Cobscook Bay. Interestingly, Maloy et al. (2003)

noted that there was a larger, though not statistically significant, decline in gamete volume fraction for *M. edulis* relative to *M. trossulus* in early September, consistent with a second spawning event for the former species. In addition, histologic analysis on the same specimens indicated that the highest proportion of individuals with undifferentiated gonads occurred from September to December for *M. trossulus* whereas few undifferentiated *M. edulis* were observed until January (Maloy personal communication). These two observations suggest that *M. edulis* is more gametogenically active during the period between September and December in Cobscook Bay. The variation in the frequency of hybrid mussels we observed is also consistent with species-specific temporal variation in spawning activity. The sharp decline in the frequency of *M. trossulus* and hybrid genotypes in our October and December samplings suggests that *M. edulis* continues to spawn after most *M. trossulus* individuals have ceased spawning.

Alternatively, differential mortality in the plankton or during metamorphosis may result in the shift in genotype frequencies we observed. A third potential hypothesis is that the increase in the frequency of *M. edulis* spat on our collectors in October and December is the result of secondary settlement. The former hypothesis requires that both *M. trossulus* and hybrid larvae experience much higher levels of mortality compared with *M. edulis* during October and November whereas the latter hypothesis requires that there be substantial genotype-specific behavioral differences among recently settled mussels. Presently, there is little comparative data on the larval physiology or behavior of *M. edulis*, *M. trossulus*, and their hybrids, so at present we favor the hypothesis that species-specific variation in spawning activity produces the temporal shift to higher frequencies of *M. edulis* spat.

Finally, our data have important implications for the potential establishment of off-bottom culture of blue mussels in Cobscook Bay. The results we have presented suggest that the deployment of spat collectors from October through December could result in >80% *M. edulis* spat. Three important caveats, however, accompany this observation. First, our sampling was confined to a single year and without additional sampling it is impossible to tell whether the increase in *M. edulis* spat late in the year will hold in other years. Second, the overall abundance of spat decreased dramatically on the collectors we retrieved in October and December so

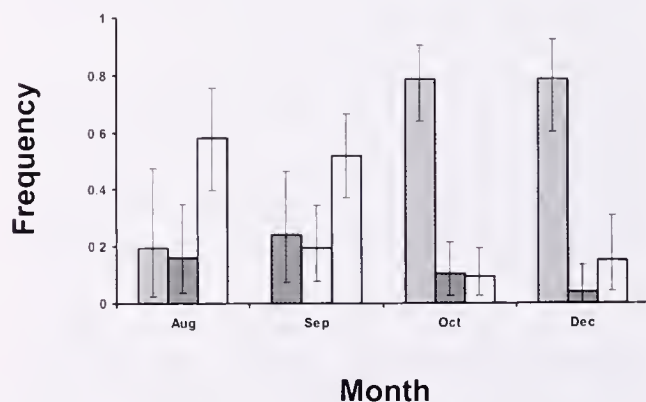


Figure 5. The average frequency of *M. edulis* (light grey), hybrid (dark grey), and *M. trossulus* (white) dilocus genotypes as a function of sampling date. Error bars represent the mean +1 standard deviation which were estimated from arcsin-transformed values and then back-transformed for clarity.

TABLE 5.

The number of mussel spat observed on top, middle, and bottom rope segments at Shackford Head, Cobscook Bay across 4 collection dates in 2000. For comparison to the data of Kenchington et al. (2002) the numbers are expressed on a per meter basis. N_{total} represents the number of all mussel spat per meter of rope. The number of *M. edulis* ($N_{M. edulis}$) versus *M. trossulus* and hybrid spat (N_{other}) was estimated using the frequency of individuals with *M. edulis* di-locus genotypes at the Glu5' and ITS markers.

Month	Depth	N_{total}	% <i>M. edulis</i>	$N_{M. edulis}$	N_{other}
August	Top	5	—		
	Middle	10	40.1	4	6
	Bottom	3	—		
September	Top	638	10.3	64	574
	Middle	560	23.9	129	431
	Bottom	260	25.2	65	195
October	Top	143	90.4	129	14
	Middle	129	79.7	103	26
	Bottom	66	74.5	50	17
December	Top	16	95.0	15	1
	Middle	35	89.6	32	4
	Bottom	118	74.2	87	31

that even Shackford Head, the best of our three sites, may not receive enough spat to seed commercially viable culture facilities. Third, spat setting later in the year are likely to realize low growth rates and potentially high mortality due to declining water temperatures. Given these caveats, reliance on spat collected late in the year would appear to be a risky strategy. The consistently high frequency of *M. trossulus* spat at all three sites and depths in September, when peak settlement occurs, would seem to preclude the development of site- or depth-specific methods for the collection of *M. edulis* spat as well as the deployment of *M. edulis* seed from external sources up to October. Thus, our results indicate that there are likely to be significant obstacles limiting the preferential collection and cultivation of *M. edulis* mussels in Cobscook Bay.

ACKNOWLEDGMENTS

This research was supported by the Maine Sea Grant program under contract 99-343 and Hatch funds provided by the US Department of Agriculture and the Maine Agriculture and Forestry Experiment Station (Project No. ME08510). The authors thank Tom Pottle, Heritage Salmon Inc. and Frank Ayres of Maine Salmon Inc. for access to their sites in Cobscook Bay and two anonymous reviewers for their helpful comments on a draft of this manuscript. Afton McGowen was supported by the Maine Research Internships for Teachers and Students Program (MERITS) sponsored by the Foundation for Blood Research. This is Maine Agricultural and Forest Experiment Station external publication #2724.

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LOCALIZED SYNCHRONOUS SPAWNING OF *MYTILUS CALIFORNIANUS* CONRAD IN BARKLEY SOUND, BRITISH COLUMBIA, CANADA

LOUIS A. GOSSELIN

Department of Biological Sciences, University College of the Cariboo, Kamloops B.C., Canada and Department of Biology, University of Victoria, Victoria B.C., Canada

ABSTRACT Although *Mytilus californianus* is a dominant intertidal species along most exposed rocky shores of the northwest coast of North America and has been studied extensively, no direct observations of spawning by this species in the field have been reported. This paper presents the first report of synchronous spawning in the field by the mussel *M. californianus*. Two synchronous spawning events were observed in Barkley Sound on the west coast of Vancouver Island, British Columbia, Canada. On both occasions several thousand mussels spawned profusely at low tide, leaving masses of gametes on their shells or concentrated in tidepools and surge channels. In the 2002 spawning event 35% of the mussels spawned, for an estimated 54,000 spawning mussels, and fertilization rate in a surge channel was ~80%. Both spawning events, however, were highly localized, occurring within 24–41 m lengths of shoreline; no mussels spawned along adjacent areas of the shore. The timing of these 2 spawning events showed no consistent relationship with tidal and lunar phases, seawater temperature or cloud cover, and mussels in adjacent parts of the shoreline did not release gametes when exposed to spawned material. Localized synchronous spawning might be common in *M. californianus*, involving a patchwork of spawning events occurring intermittently over periods of a few months or throughout the year. This could provide the benefits of synchronous spawning as well as the advantages of the continuous production of recruits.

KEYWORDS: life cycle, reproduction, fertilization rate, gametes, broadcast spawning, *Mytilus*

INTRODUCTION

Among aquatic organisms, the synchronous release of gametes or larvae by many individuals of a population can provide increased fitness (see review by Olive 1992) by enhancing fertilization rates in broadcast spawners (Pennington 1985, Babcock 1995), taking advantage of hydrodynamic conditions that optimize fertilization or dispersal (Pennington 1985, Olive 1992), reducing larval mortality by predator satiation (Harrison et al. 1984, Slattery et al. 1999), and synchronizing the timing of planktotrophic larval development with that of algal blooms which the larvae use as food (Starr et al. 1990). Knowledge of whether a species spawns synchronously is therefore important in elucidating its population dynamics. In addition, an understanding of how environmental factors influence the timing of spawning can help predict future spawning events or determine how changes in environmental conditions can affect reproductive success (Minchin 1992, Olive 1992, Watson et al. 2000).

Synchronous spawning has most commonly been reported in anthozoans (Harrison et al. 1984, Babcock et al. 1986, 1992, Brazeau & Lasker 1989) and various echinoderms (Pearse et al. 1988, Babcock et al. 1992, Lamare & Stewart 1998) but also occurs in other marine invertebrates such as bivalves (Babcock et al. 1992, Minchin 1992, Grant & Creese 1995), gastropods (Counihan et al. 2001) and polychaetes (Caspers 1984, Hardege 1999, Watson et al. 2000). Sightings of natural spawning, however, are most often chance observations and, as a result, many species that do spawn synchronously may simply not yet be known to do so. This paper reports the first observations of synchronous spawning by the mussel *Mytilus californianus*.

Mytilus californianus is a dominant intertidal species on exposed rocky shores along the northwest coast of North America, forming extensive mussel beds from British Columbia to California (Suchanek 1981). Studies based on gonad condition or laboratory spawning indicate that this species is capable of spawning throughout the year (Young 1945, Kelly et al. 1982, Dittman & Robles 1991) and that the peak spawning period in British Colum-

bia occurs in the summer (Kelly et al. 1982). A study of male gonad development in *M. californianus* in British Columbia concluded that many individuals are trickle-spawners (Kelly et al. 1982). However, no direct observations of spawning by this species in the field have been reported. This paper (1) reports 2 synchronous spawnings by *Mytilus californianus*, one in 1992 and the other in 2002, (2) quantifies the spawning rate and fertilization rate for the 2002 event, and (3) examines the relation of these 2 events with tidal, lunar, seawater temperature and cloud cover conditions.

METHODS AND RESULTS

The 2 spawning events described herein occurred in Barkley Sound on the west coast of Vancouver Island, British Columbia, Canada, and were discovered while visiting the sites at low tide. Tide levels for periods when the spawning events occurred were obtained from the Canadian Hydrographic Service (1991, 2001) tide tables. Surface seawater temperature data for Cape Beale (48°47'20"N, 125°12'95"W) and Amphitrite Point (48°55'25"N, 125°32'50"W), for 1992 and 2002, were obtained from the Fisheries and Oceans Canada online database for BC lighthouses (http://www-sci.pac.dfo-mpo.gc.ca/osap/data/SearchTools/SearchLighthouse_e.htm). Amphitrite Point data was used for 2002 because seawater temperature measurements at Cape Beale ended in 1998.

1992 Spawning Event

The first spawning event was observed at Kirby Point (48°50'85"N, 125°21'40"W) on 22 June 1992. A large number of mussels had spawned profusely and several tidepools were clouded with sperm and lined with a coating of orange oocytes. Mussels outside of tidepools had masses of gametes on their shells near the siphons. Spawning was limited to a 24 m section of shoreline in which the mussel bed ranged in width from 3.0 m

to 14.5 m; the surface area within which spawning occurred was $\sim 214 \text{ m}^2$. No spawning mussels were found within 100 m on either side of the spawning area. In addition, to determine if spawning was stimulated by exposure to spawned material from other mussels, 3–4 l of water from a tidepool with an abundance of spawned material was transferred to each of 3 other small tidepools several metres away, in which no spawned material was apparent. Each tidepool contained 10–30 mussels. No spawning was observed over the following 2 h in the 3 tidepools receiving spawned material. This spawning event occurred at a time when the tidal amplitude was modest (Fig. 1A), with the moon in its last quarter. The weather that morning was sunny, and surface seawater temperature had been increasing for over 2 weeks (Fig. 1B).

2002 Spawning Event

The second spawning event was observed during a collecting trip to the intertidal zone at Cape Beale on the morning of 8 August 2002. Large numbers of emersed mussels were found with masses of gametes at the margin of their shell near the siphons. The water in several small tidepools as well as in a surge channel running parallel to the shoreline was yellowish-brown from the high densities of gametes. This spawning event occurred during very high amplitude tides (Fig. 2A) at new moon. The weather that morning was overcast, and surface seawater temperature had

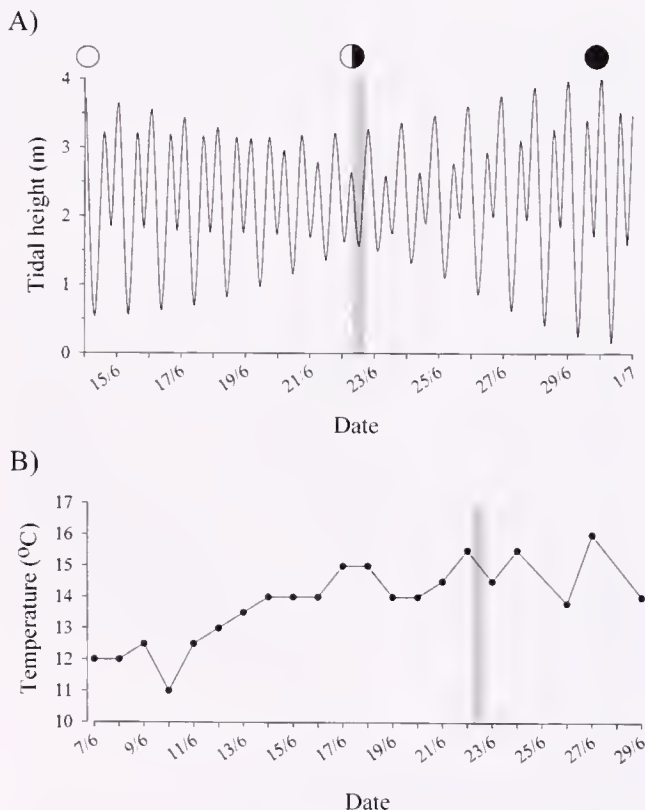


Figure 1. Environmental conditions prior to and after the synchronous spawning event of *Mytilus californianus* in 1992. The dashed areas in each graph represent the time of the spawning event. (A) Tidal heights and lunar phases; (B) surface seawater temperatures measured at the Cape Beale lighthouse.

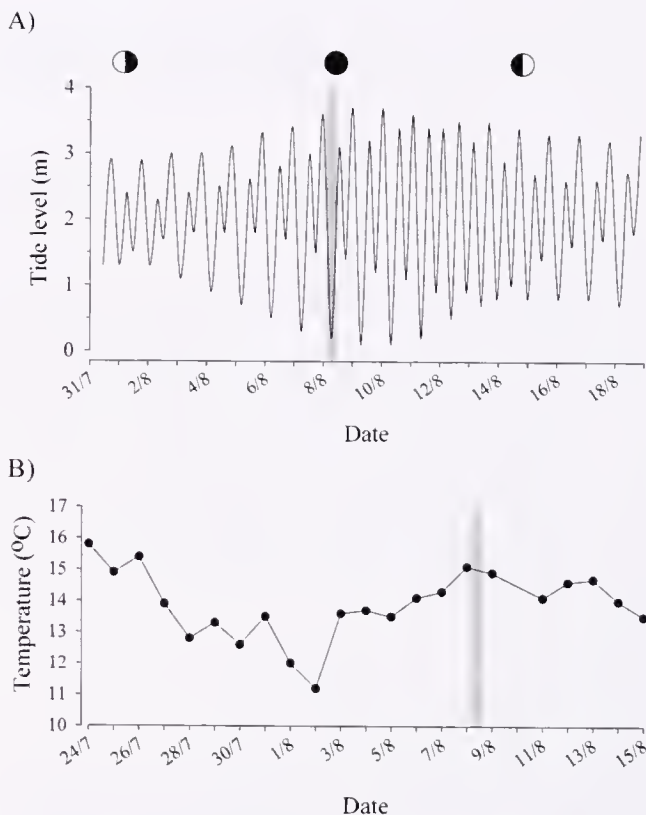


Figure 2. Environmental conditions prior to and after the synchronous spawning event of *Mytilus californianus* in 2002. The dashed areas in each graph represent the time of the spawning event. (A) Tidal heights and lunar phases; (B) surface seawater temperatures measured at the Amphitrite Point lighthouse.

been relatively stable over the 5d preceding the spawning event, with a slight increase on the day of spawning (Fig. 2B). To further characterise the event 4 sets of observations and measurements were taken.

Mussel Densities and Proportion of Mussels that Spawning

To quantify mussel densities and the proportion of mussels that spawned, observations were made along 2 separate transects running parallel to the shoreline within the mussel bed. Each transect consisted of five $25 \times 25 \text{ cm}$ quadrats set at 1 m intervals. In each quadrat I counted the total number of visible mussels and the number of mussels having spawned to determine the proportion of mussels that had spawned. Spawned gametes, when present, were located at the margin of the shell near the siphons or, when located elsewhere on the shell, a shiny slime coating could be observed running from the shell margin to the spawned material. Mussels were not dislodged for these observations; if small juvenile mussels were present deeper within the mussel bed they would not have been detected. *Mytilus californianus* densities in individual quadrats within the mussel bed ranged from 392 to 984 mussels m^{-2} and averaged 678 mussels m^{-2} (Table 1). Although densities varied among the 2 transects, the proportion of *M. californianus* that had spawned was consistent, averaging $\sim 35\%$ in each transect. No spawned material was present on low intertidal mussels that were exposed to wave wash, but the abundant gametes in the nearby water indicated that many of these had also spawned.

TABLE 1.

Mytilus californianus. Densities and percent of mussels found to have spawned at Cape Beale on 8 August 2002.

	Mussel Density m^{-2} (\pm SD)	% Spawned (\pm SD)
Transect 1	520.0 \pm 119.6	33.0 \pm 15.4
Transect 2	836.8 \pm 122.45	36.1 \pm 4.8
Overall	678.4 \pm 202.2	34.51 \pm 10.9

Smallest Spawning Mussel

The mussel bed was closely examined for 15 minutes to search for and measure the smallest mussels with spawned material. The smallest spawning mussel measured 28 mm shell length, which is within the reported size range (25–30 mm) at which *Mytilus californianus* reaches maturity (Suchanek 1981). Several 30–50 mm mussels had also spawned. Mussel sizes at that site ranged from 10.2 mm to 150.0 mm, with most mussels measuring 60 mm to 120 mm. Few were smaller than 30 mm. The spawning event thus appears to have included all mussel sizes present in the bed.

Number of Mussels Spawning

Mussels with spawned gametes were found only along a 41 m section of shoreline. The width of the mussel bed along that section of shoreline ranged from 1.8 m to 14.2 m, for a surface area of the bed where spawning occurred of $\sim 231 \text{ m}^2$. No spawning mussels were found over $\sim 100 \text{ m}$ on either side of the spawning area. Using an average mussel density of 678.4 m^{-2} and an average of 34.51% of mussels spawning (Table 1), the total number of mussels spawning in this event is estimated at 54,080 individuals.

Oocyte and Egg Density and Percent Fertilization in a Surge Channel

The combined density of oocytes and eggs as well as percent fertilization was determined by collecting a 60 ml sample of seawater from a surge channel formed by a rocky outcrop running parallel to the shoreline. The swell entering the channel caused continuous and substantial turbulence, thoroughly mixing the water throughout the channel. Oocytes and eggs were counted in the laboratory within five 0.1 ml subsamples. In addition, I estimated the proportion of oocytes that had been fertilized from the proportion that had undergone cleavage at 1:15 PM the same day, approximately 5–10 h after they had been spawned. Combined oocyte and egg density in the channel running parallel to the mussel bed was $885 \pm 80.1 \text{ ml}^{-1}$ (average \pm SD). Based on measurements of length, width and depth of the channel, the volume of water in the channel at the time the sample was taken was estimated to be $\sim 60,800 \text{ l}$. Using 885 eggs ml^{-1} as an estimate of density throughout the channel, the water in that channel would have contained $\sim 5.4 \times 10^{10}$ oocytes and eggs. In the sample returned to the laboratory, $80.7 \pm 12.3 \%$ (average \pm SD) of these developed at least to the 2-cell stage. Using 80.7% as an estimate of fertilization rate, the channel would have contained $\sim 4.3 \times 10^{10}$ fertilized eggs. This constitutes the output of only the lower part of the mussel bed, as approximately two-thirds of the mussel bed was above the splash zone at the time of sampling and the gametes produced by those mussels were not suspended until later by the rising tide.

DISCUSSION

Localized Synchronous Spawning in *Mytilus californianus*

Mytilus californianus have the capacity to spawn at any time of the year (Young 1945, Kelly et al. 1982, Smith & Strehlow 1983, Dittman & Robles 1991). It has also been suggested, based on observations of juvenile recruitment throughout the year, that *M. californianus* spawns continually releasing small amounts of gametes throughout the year (Suchanek 1981) and a study of gonad condition in male mussels revealed that about half of the males are probably 'trickle' spawners (Kelly et al. 1982). None of these conclusions, however, are based on observations of spawning in the field. Despite its widespread occurrence and ecological importance along the rocky shores of the northwest coast of North America, there are no reports of *M. californianus* spawning in the field. In the present study, more than 50,000 mussels spawned in the 2002 event. Mussel densities were not quantified in 1992; however, assuming an average density similar to that observed at Cape Beale in 2002 of $678.4 \text{ mussels m}^{-2}$, the total number of mussels in that section of mussel bed would have been $\sim 145,000$ individuals. The number of mussels spawning in the 1992 event was thus likely in the order of several thousand. These findings reveal that events in which large numbers of individuals spawn synchronously do occur in this species. This is the first report of synchronous spawning in *M. californianus* and the first such report for a species in the genus *Mytilus*.

The 80.7% fertilization rate observed during the 2002 spawning event is consistent with the expectation that synchronous spawning in a population favours high fertilization rates (Pennington 1985, Olive 1992, Babcock 1995). This particularly high fertilization rate, however, was possibly due to the retention and continuous mixing within the limited volume of the surge channel, as fertilization rate should decrease with dilution of the gametes (Pennington 1985, Levitan et al. 1992). Particularly intriguing, however, is the fact that both spawning events were very localized. While a high proportion of mussels in a given area of shoreline spawned, mussels in adjoining areas of the shoreline did not. The boundary between spawning and non-spawning areas of the mussel bed occurred over 1–2 m, and there was no apparent physical difference in the shoreline or in the size or health of the mussels between areas with and without spawning mussels.

In addition, it is not clear why the mussels spawned at low tide; why not release the gametes at high tide when fully submerged? One might expect the viability of gametes released at low tide to be decreased due to exposure to air and ultraviolet radiation. Given the rarity of observations of spawning by *Mytilus californianus* in the field, it may be that spawning usually takes place at high tide and that low tide spawning events are rare occurrences.

Factors Influencing Spawning Synchrony

Several factors have been reported to induce or synchronize spawning in marine invertebrates, including tidal and lunar phases (Caspers 1984, Babcock et al. 1986, Slatery et al. 1999, Watson et al. 2000), changes in seawater temperature (Minchin 1992), mechanical stimulation by water flow (Desrosiers & Dubé 1993) or by wave action (Young 1945), clement weather (Watson et al. 1986), and water-borne chemicals released by other spawning individuals (Young 1945, Atkinson & Atkinson 1992, Hardege

1999) or by phytoplankton (Breese & Robinson 1981, Smith & Strehlow 1983, Starr et al. 1990, Parsons et al. 1992). The present study therefore documented tidal and lunar phases, seawater temperature (measured 1–3 km from the spawning sites) and weather (cloud cover) to determine if the 2 spawning events occurred under similar conditions. None of these factors, however, were the same on both spawning events. Finally, water transferred from tidepools with spawned gametes to tidepools in which mussels had not spawned did not induce spawning, although it is possible that those mussels had spawned on previous days and thus were unable to spawn that morning.

Ecological Significance of Synchronous Spawning by *Mytilus californianus*

Observations of the two spawning events described herein were serendipitous. I have frequently visited various rocky intertidal sites colonized by *Mytilus californianus* in Barkley Sound over the last 14 years but only observed mussels spawning on these 2 occasions. These observations nevertheless reveal that synchronous spawning of several thousand *M. californianus*, and by as much as 35% of the individuals of a mussel bed, does occur. It may be that such events are not rare, but happen mostly at night or when

the mussels are submerged, and therefore spawning goes unnoticed. If localized synchronous spawning is common in *M. californianus*, reproduction in this species could involve a patchwork of spawning events occurring intermittently over periods of a few months or throughout the year, which would be consistent with Suchanek's (1981) observations of recruitment throughout the year on the northern coast of Washington State. This strategy would allow the species to benefit from the advantages of synchronous spawning as well as from the continued production of recruits allowing the opportunistic colonization of habitats as they become available, free space being a limiting resource on these exposed rocky shores, made periodically available by disturbance events such as storms and impacts by floating logs (Dayton 1971, Paine 1974).

ACKNOWLEDGMENTS

Thanks to the director and staff of the Bamfield Marine Sciences Centre for research facilities and support. Thanks to E. G. Boulding, C. Robles and two anonymous reviewers for reading and commenting on earlier versions of the manuscript and to Carla Gibbons for assistance in the field. Financial support was provided by an NSERC Discovery grant and by SAC grants from UCC to LAG, and an NSERC USR Award to C. Gibbons.

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MINI-REVIEW: DISTRIBUTION OF THE MEDITERRANEAN MUSSEL *MYTILUS GALLOPROVINCIALIS* (BIVALVIA: MYTILIDAE) AND HYBRIDS IN THE NORTHEAST PACIFIC

MARJORIE J. WONHAM*

University of Washington, Department of Zoology, Box 351800, Seattle, WA, USA 98195-1800

ABSTRACT The non-native Mediterranean mussel *Mytilus galloprovincialis* is broadly established in the northeast Pacific. Until recently, the coast north of Humboldt Bay, California, USA, was not considered a major zone of sympatry and hybridization with the native sibling species *M. trossulus*. However, *M. galloprovincialis* has been introduced in Washington, USA, and British Columbia, Canada, for aquaculture, has been collected from ballast water in-bound to Oregon, USA, and is now reported widely in Puget Sound, Washington. Here I review published reports of *M. galloprovincialis* alleles in the Northeast Pacific, including recent data showing that these alleles are more widespread and abundant in Washington than previously known. These results indicate the presence of a major zone of sympatry and hybridization in Washington waters that may be contiguous with the California zone. Because *M. galloprovincialis* has likely been introduced to the region on multiple occasions via multiple routes, it is unlikely that a sole source can be identified. Factors influencing the success and impacts of this now widespread invader remain to be investigated.

KEY WORDS: introduced marine species, hybridization, Pacific Northwest, *Mytilus galloprovincialis*

INTRODUCTION

The marine mussel genus *Mytilus* includes a complex of 3 sibling species, *M. edulis* L. 1758, *M. trossulus* Gould 1850, and *M. galloprovincialis* Lamarck 1819. All three are now globally widespread and form hybrid zones where they overlap (McDonald et al. 1991, Sarver & Foltz 1993, Hilbish et al. 2000). The role of human activity in establishing the global but disjunct distributions of these species has been addressed in terms of genetic evidence, fossil evidence, and the available introduction pathways. (e.g., McDonald et al. 1991, Carlton 1999, Daguin & Borsa 2000, Hilbish et al. 2000)

Certain features of *Mytilus* species make them likely candidates for human-mediated introduction: their planktonic larval stage allows them to be passively transported in the ballast water of commercial ships (e.g., Carlton & Geller 1993), byssal threads produced by juveniles and adults allow transport on hard substrata including ship and boat hulls (Carlton & Hodder 1995, Apte et al. 2000), and their palatability and relative ease of culture has led to their widespread introduction for aquaculture (Heath et al. 1995, Couturier 2003 and references therein).

M. galloprovincialis is the most widely distributed of the three sibling species, and recent genetic analyses distinguish among multiple hypotheses for its origin and spread (Daguin & Borsa 2000). In the Northeast Pacific, *M. galloprovincialis* ranges from Mexico to central California, and recent surveys confirm its presence throughout the inner waters of Washington State (Suchanek et al. 1997, Anderson et al. 2002, present study). The origin, timing, and pathways of its introduction to Washington remain unclear, and it has likely been introduced on multiple occasions. Here, I summarize recent genetic analyses of *Mytilus* spp. distributions globally and in the northeast Pacific, provide additional sampling results from Washington State waters, review our current understanding of the *M. galloprovincialis* introduction to the region, and identify ecologic questions to be addressed concerning this introduction.

Global *Mytilus* Distributions

Because *Mytilus* sibling species and their hybrids cannot reliably be distinguished with morphologic characters alone, genetic analysis is required to determine their distributions and origins. *M. edulis* and *M. galloprovincialis* are the most closely related and *M. trossulus* is the most divergent according to genomic DNA (Beynon & Skibinski 1996, Eirin-Lopez et al. 2002, Martínez-Lage et al. 2002) and mtDNA analysis (Rawson & Hilbish 1995b, Quesada et al. 1998, Geller 1999, Hilbish et al. 2000). Two additional studies report evidence that may suggest that *M. galloprovincialis* is the most divergent of the three species (Wenne & Skibinski 1995, Varvio et al. 1988). However, the mtDNA-based species determinations in one study (Wenne & Skibinski 1995) may have been confounded by sex-specific differences, and the other study stated clearly that their allozyme analysis should not be taken to indicate relative relatedness among the species (Varvio et al. 1988).

The three sibling species have distinct but overlapping distributions. *M. trossulus* is circumpolar in the north Pacific, northwest Atlantic, and Baltic, but has not been unambiguously identified in the southern hemisphere (McDonald et al. 1991, Hilbish et al. 2000). *M. edulis* is found in the northeast and northwest Atlantic, and *edulis*-like mussels are reported from South America (McDonald et al. 1991). *M. edulis* has also been introduced to British Columbia for aquaculture (Heath et al. 1995). *M. galloprovincialis* is distributed throughout the Mediterranean and into the northeast Atlantic, with additional populations in California, Japan, South Africa, Australasia and Chile (McDonald et al. 1991, Sanjuan et al. 1997, Daguin & Borsa 2000, Hilbish et al. 2000). To account for its disjunct distribution, *M. galloprovincialis* has been proposed variously to be endemic to the Mediterranean and introduced in the north and south Pacific (Barsotti & Meluzzi 1968, McDonald et al. 1991, Carlton 1999), possibly endemic to the south Pacific (Koehn 1991, McDonald et al. 1991), and endemic to both the Pacific and Mediterranean (Sanjuan et al. 1997). These alternate hypotheses are comprehensively reviewed by Daguin and Borsa (2000) and Hilbish et al. (2000); the key points are summarized here.

Northern hemisphere populations of *M. galloprovincialis* in the Atlantic and the Mediterranean can be distinguished by allozyme (Quesada et al. 1995b, Sanjuan et al. 1997) and mtDNA analysis

*Corresponding author. E-mail: mwonham@ualberta.ca

(Quesada et al. 1995a, Quesada et al. 1998, Ladoukakis et al. 2002). The Californian population is more closely related to the Mediterranean than to the Atlantic population in allozyme (McDonald & Koehn 1988, Sanjuan et al. 1997) and genomic DNA studies (Daguin & Borsa 2000). In the southern hemisphere, *Mytilus* sp. appear in fossil beds and middens, and genomic DNA analysis indicates that present-day populations are closest to, but readily distinguished from, Mediterranean populations (Daguin & Borsa 2000, Hilbish et al. 2000).

The emerging evolutionary and historical picture that appears most consistent with genetic and geological data is that the genus *Mytilus* evolved in the north Pacific, that the *M. trossulus* stock migrated ~3.5 mya through the Bering Strait to the north Atlantic where the *M. edulis* stock arose, and that *M. galloprovincialis* subsequently diverged from *M. edulis* in the Mediterranean (Vermeij 1991, 1992; Daguin and Borsa 2000; Hilbish et al. 2000). Subsequently, both *M. galloprovincialis* and *M. edulis* migrated to the southern hemisphere (Vermeij 1991, 1992, Daguin & Borsa 2000, Hilbish et al. 2000). Since these early natural dispersal events, more recent translocations have occurred to California, southern Africa, and the northwest Pacific (Wilkins 1983, Grant & Cherry 1985, Lee & Morton 1985, McDonald & Koehn 1988, Inoue et al. 1997, Sanjuan et al. 1997, Daguin & Borsa 2000). Because none of these introductions is known to have been intentional, they most likely occurred accidentally via commercial shipping transport. *Mytilus* species may also have been recently transported to the southern hemisphere by shipping; this remains to be determined.

Northeast Pacific *Mytilus*

The broad-scale distribution of *Mytilus* species along the Pacific coast of North America is well established. *M. trossulus* is currently found from Alaska south to Monterey Bay, California (~36°N) and *M. galloprovincialis* from Mexico north to Humboldt Bay, California (~38°N). An apparently stable hybrid zone extends between San Diego (~32°N) and Humboldt Bay (McDonald & Koehn 1988, McDonald et al. 1991, Sarver & Foltz 1993). The congener *M. californianus* is a morphologically, genetically, and ecologically distinct species that is found on exposed shores along the entire coast (Sarver & Foltz 1993); it is not treated further here.

Until recently, only a handful of *M. galloprovincialis* and hybrids had been collected from Oregon to British Columbia, and the coastline north of Humboldt Bay was not considered a major zone of sympatry and hybridization (Heath et al. 1995, Rawson & Hilbish 1995a, Suchanek et al. 1997, Rawson et al. 1999). However, *M. galloprovincialis* has been widely introduced for aquaculture in Washington and British Columbia, and *M. edulis* to a lesser extent in British Columbia (Brooks 1991, Heath et al. 1995, Hilbish 1999, Anderson et al. 2002). In addition, *M. galloprovincialis* has been identified in ballast water loaded in Japan and scheduled for discharge in Oregon (Carlton & Geller 1993, Geller et al. 1994, Suchanek et al. 1997). Given the large scale of both aquaculture and ship-mediated invasion pathways in the Pacific Northwest (Wonham & Carlton 2004), we might expect *M. galloprovincialis* (and possibly *M. edulis*) to be more widely distributed in these waters. Indeed, recent surveys of Puget Sound and the Strait of Juan de Fuca, Washington, indicate that *M. galloprovincialis* alleles are distributed broadly in the south and central sound and in the Strait of Juan de Fuca (Anderson et al. 2002, present study). Along the outer coast, *M. galloprovincialis* is reported from north-

ern California and Oregon, but seems to be largely absent from Washington (Suchanek et al. 1997, Brooks 1991). Northeast Pacific records of *M. galloprovincialis* and hybrids are detailed here by province and state.

British Columbia

Heath et al. (1995) sampled 12 sites on Vancouver Island and the adjacent mainland (Fig. 1, Table 1). They found non-native alleles belonging to either *M. galloprovincialis* or *M. edulis* at five sites from Yellow Island to Victoria. Because mussel aquaculture is continuing to develop in BC, new surveys in this region using species-specific markers are welcome.

Washington

The subtidal distribution of *M. galloprovincialis* alleles in Washington extends throughout the Strait of Juan de Fuca, the North Puget Trough, and Puget Sound (Fig. 2, see Table 1). *M. galloprovincialis* was identified as early as 1988 in the eastern strait (Brooks 1991), and extensive sampling in the straits and Puget Sound indicates it is now ubiquitous in this region (Anderson et al. 2002, present study). It has been found in the East, Main, and South Basins of Puget Sound, but not in Hood Canal, and appears less frequently in Northern Puget Trough (see Fig. 1, Table 1). Brooks (1991) found no *M. galloprovincialis* on the outer coast of Washington; one hybrid was reported from Tatoosh Island by Suchanek et al. (1997) but none was found there in recent sampling (present study; see Fig. 1, Table 1).

In this study, I made 16 collections totaling 390 individual *Mytilus* at 15 Washington sites from 1997 to 2000 (see Table 1). Subtidal mussels were collected by hand from public docks and marinas and one aquaculture farm. Ten of the docks and marinas were sampled during a rapid-assessment survey for introduced marine species (Cohen et al. 1998), and an additional four were sampled along the northern Olympic peninsula (see Fig. 1, Table 1). At each site, up to 15 mussels were collected by hand from underneath floating docks. Mussels were selected haphazardly, but because they were collected as visible and accessible among other fouling species, they tended to be large (all 3–10 cm long). Mussels in the same size range were also collected from suspended culture ropes at Taylor United Shellfish farm, Shelton, Washington. These included both cultured mussels (i.e., *M. galloprovincialis* seeded from hatchery stock) and natural-set mussels (see Table 1). Intertidal mussels (1–3 cm shell length, representing the largest mussels at the site) were collected from the rocky shores of Tatoosh Island at the entrance of the Strait of Juan de Fuca, and Saddlebag Island in Padilla Bay (see Fig. 1, Table 1). All specimens were stored at -10°C until identification.

Mussels were dissected and DNA was extracted from 0.5 g of gonad tissue following Geller et al. (1994). Polymerase chain reaction (PCR) amplification followed Suchanek et al. (1997) using the species-specific primers developed by Inoue et al. (1995). The PCR product was run on a 2% agarose gel: individuals homozygous for this marker exhibit a single band and heterozygotes exhibit bands of both parental species (Inoue et al. 1995, Suchanek et al. 1997, Wonham 2001). All heterozygotes are *M. galloprovincialis* x *M. trossulus* hybrids, whereas homozygotes may represent either pure or introgressed genomic DNA strains. Although introgression seems to be limited in these populations (Rawson et al. 1999), heterozygote frequencies nonetheless probably underestimate the relative abundance of hybrids. Reference DNA samples

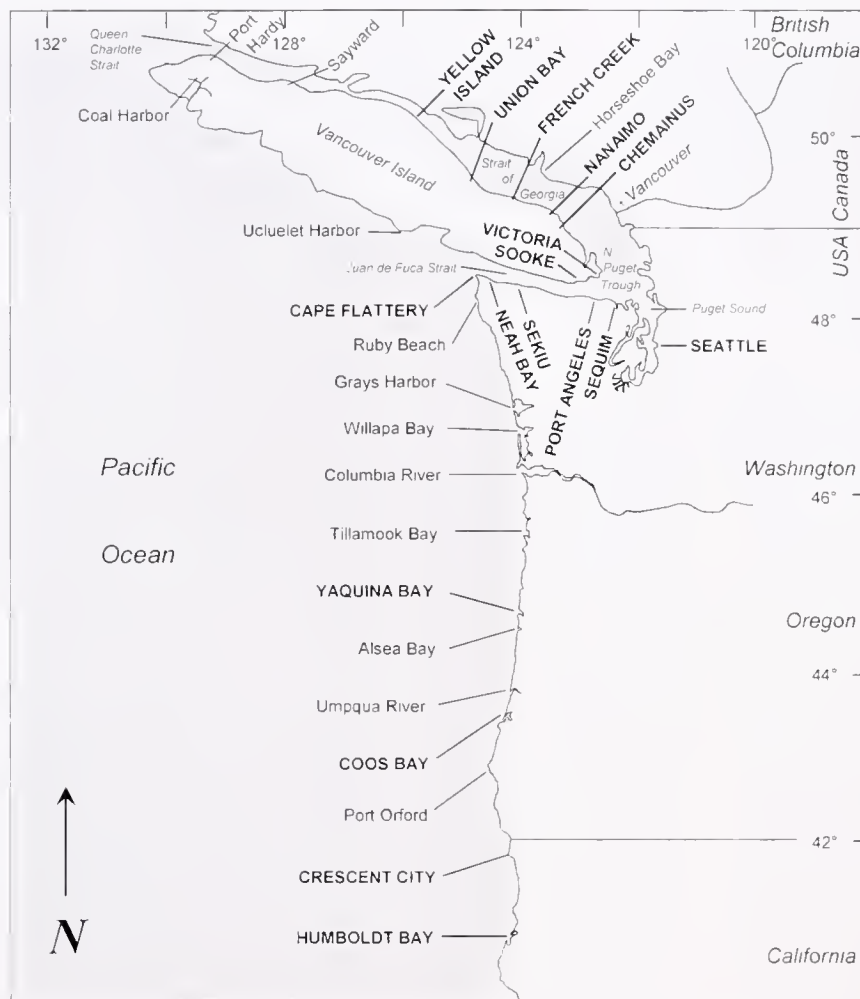


Figure 1. *Mytilus* spp. sampling sites along the Pacific coast. Sites where *M. galloprovincialis* genes were detected indicated in bold capitals. Sites where only *M. trossulus* genes were detected indicated in regular type. Landmarks indicated in italics. For details see Table 1.

for each species were provided by J. Mitton, University of Colorado. For further collection and analysis details see Wonham (2001).

I found *M. galloprovincialis* genes in subtidal mussel samples throughout Puget Sound, the Strait of Juan de Fuca, and northern Puget Trough. Homozygote *M. galloprovincialis* and heterozygotes were present at 4/4 marinas along the Strait of Juan de Fuca, and 6/8 marinas in Puget Sound (Figs. 1 and 2, see Table 1). Together they averaged of 22% of mussels in both regions (range 0% to 50% in the strait and 8% to 54% in the sound, excluding aquaculture samples). They were present at only one site in northern Puget Trough (see Fig. 1, Table 1).

This Puget Sound and Trough distribution of *M. galloprovincialis* is largely consistent with that found by Anderson et al. (2002), who sampled similar locations. Differences are that I found *M. galloprovincialis* alleles at Steilacoom and Elliott Bay Marinas where they did not, but not at Seattle (Harbor Island Marina) or Tacoma (Ole and Charlie's Marina), where they did. I take these minor differences between studies to indicate local spatial heterogeneity in mussel species distributions: with larger sample sizes at identical sites the results would likely be consistent.

The frequency of *M. galloprovincialis* genes (22%) exceeds previous estimates for Puget Sound by approximately 4-fold

(Brooks 1991, Suchanek et al. 1997, Anderson et al. 2002, cultured mussels excluded from all studies). Because my collections and those of Anderson et al. (2002) were made in the same year, the difference does not reflect a temporal change. Instead, I suggest that it may reflect differences in the size of sampled mussels. The subtidal mussels I collected were 3–10 cm in shell length, whereas those analyzed by Anderson et al. (2002) were as small as 0.5 cm. Further evidence for a difference in sizes is found in the detailed analysis by Anderson et al. (2002) of mussels at one site, where they found that smaller mussels had predominantly *M. trossulus* alleles and larger ones had predominantly *M. galloprovincialis* alleles. The potentially larger size of *M. galloprovincialis* has implications for the invader's fecundity and spread relative to its native sibling species.

Although *M. galloprovincialis* was readily found at subtidal sites, I found none in intertidal samples from Tatoosh Island. Because *M. galloprovincialis* is common at other intertidal sites in the Pacific and Atlantic (Sarver & Foltz 1993, Quesada et al. 1995b, Rawson & Hilbish 1995a, Wilhelm & Hilbish 1998), it seems likely that it will also invade this habitat in Washington waters. On the other hand, at one site in Posjet Bay, Russia, McDonald et al. (1991) found exclusively *M. trossulus* intertidally and exclusively *M. galloprovincialis* subtidally. Only one intertidal hybrid has

TABLE 1.

Mussel *Mytilus* spp. records on the Pacific coast of North America from Queen Charlotte Strait, British Columbia, to Humboldt Bay, California. For each site, proportion of, homozygous *M. galloprovincialis* alleles, Mg, homozygous *M. trossulus* alleles, Mt, and heterozygous hybrids, Mg × Mt, given. N, number of individuals sampled. Sites in bold are those with *M. galloprovincialis* or hybrid individuals; p, genes present but not quantified; nd, data not provided in original study; –, marker could not distinguish heterozygotes. Latitude (°N), longitude (°W), and sampling date (m/yy) given for intertidal and subtidal mussel *Mytilus* spp. collection sites in the present study; sites indicated with * were sampled during a rapid-assessment survey for introduced marine species in Puget Sound (Cohen et al. 1998).

Location Site	Mg	Mg × Mt	Mt	N	Source
British Columbia outer coast					
Vancouver Island					
Coal Harbour	0.00	0.00	1.00	22	Heath et al. 1995 ^a
Ucluelet Harbour	0.00	0.00	1.00	23	Heath et al. 1995
Strait of Georgia & Queen Charlotte Strait					
Vancouver Island					
Port Hardy	0.00	0.00	1.00	29	Heath et al. 1995
Sayward	0.00	0.00	1.00	35	Heath et al. 1995
Yellow Island	0.00	0.06	0.94	79	Heath et al. 1995
Union Bay	0.04	0.00	0.96	26	Heath et al. 1995
French Creek	0.06	0.03	0.91	35	Heath et al. 1995
Nanaimo	0.03	0.03	0.93	29	Heath et al. 1995
Chemainus	0.14	0.00	0.86	29	Heath et al. 1995
Mainland					
Horseshoe Bay	0.00	0.00	1.00	25	Heath et al. 1995
North Puget Trough					
Bellingham Bay*	0.18	0.09	0.73	11	Present study (9/98) (48°76', 122°49')
	0.00	–	1.00	22	Anderson et al. 2002 ^c
Saddlebag Island, Padilla Bay	0.00	0.00	1.00	1	Present study (7/97) (48°32', 123°33')
Anacortes					
Anacortes	0.00	–	1.00	32	Anderson et al. 2002
Anacortes City Pier*	0.00	0.00	1.00	10	Present study (9/98) (48°31', 122°36')
San Juan Island					
Argyle Creek	0.00	0.00	1.00	6	Suchanek et al. 1997
Eagle Cove	0.00	0.00	1.00	6	Suchanek et al. 1997
Friday Harbor Laboratory*	0.00	0.00	1.00	2	Present study (9/98) (48°32', 123°01')
Puget Sound-East Basin					
Whidbey Is. (E)					
Deception Pass Marina	0.00	–	1.00	30	Anderson et al. 2002
Oak Harbor Crescent Harbor	0.00	–	1.00	32	Anderson et al. 2002
Penn Cove	0.25	0.10	0.65	20	Suchanek et al. 1997
	0.00	0.00	1.00	563	Brooks 1991 ^d
Holmes Harbor (Honeymoon H.)	0.01	0.02	0.97	200	Brooks 200
Holmes Harbor (Freeland)	0.00	–	1.00	30	Anderson et al. 2002
Possession Point	0.00	–	1.00	28	Anderson et al. 2002
Puget Sound-Hood Canal					
Hood Canal	0.00	0.00	1.00	54	Brooks 1991
Lilliwaup	0.00	–	1.00	30	Anderson et al. 2002
Seal Rock, Brannon	0.00	–	1.00	54	Anderson et al. 2002
Potlatch State Park	0.00	–	1.00	30	Anderson et al. 2002
Twanoh State Park	0.00	–	1.00	32	Anderson et al. 2002
Belfair State Park	0.00	–	1.00	26	Anderson et al. 2002
Puget Sound-Main Basin					
Whidbey Is. (W)					
Fort Casey	p	nd	nd	nd	Brooks 2000
Keystone Ferry	0.00	–	1.00	32	Anderson et al. 2002
Mutiny Bay	0.00	–	1.00	30	Anderson et al. 2002
Edmonds					
Edmonds Marina*	0.07	0.20	0.73	15	Present study (9/98) (47°49', 122°23')
Edmonds	0.19	–	0.82	26	Anderson et al. 2002
Seattle					
Seahurst County Park	0.19	–	0.81	32	Anderson et al. 2002
Shilshole Bay	0.12	–	0.88	68	Anderson et al. 2002
Elliott Bay Marina*	0.00	0.15	0.85	13	Present study (9/98) (47°38', 122°22')
	0.00	–	1.00	66	Anderson et al. 2002
Seattle Pier 91	0.00	–	1.00	28	Anderson et al. 2002

TABLE 1.
continued

Location	Site	Mg	Mg × Mt	Mt	N	Source
	West Seattle	0.00	—	1.00	24	Anderson et al. 2002
	Harbor Island Marina*	0.00	0.00	1.00	13	Present study (9/98) (47°35', 122°22')
Des Moines						
	Saltwater State Park	0.04	—	0.96	28	Anderson et al. 2002
	Des Moines Marina*	0.14	0.14	0.71	14	Present study (9/98) (47°24', 122°19')
Tacoma						
	Point Defiance	0.07	—	0.93	28	Anderson et al. 2002
	Ole & Charlie's Marina*	0.00	0.00	1.00	9	Present study (9/98) (47°12', 122°29')
Manchester						
	Manchester	p	nd	nd	nd	Brooks 2000
	Manchester State Park	0.00	—	1.00	48	Anderson et al. 2002
Silverdale, Dyes Inlet		0.35	—	0.65	126	Anderson et al. 2002
		0.18	0.67	0.15	54	Brooks 1991
	Poulsbo, Liberty Bay	0.00	—	1.00	32	Anderson et al. 2002
	Kingston	p	nd	nd	nd	Brooks 2000
Puget Sound-South Basin						
	Steilacoom Marina*	0.00	0.25	0.75	12	Present study (9/98) (47°10', 122°36')
		0.00	—	1.00	62	Anderson et al. 2002
Budd Inlet						
	Skookum Bay	0.00	0.00	1.00	7	Suchanek et al. 1997
	Tolmie State Park	0.00	—	0.00	44	Anderson et al. 2002
	Boston Harbor Marina*	0.18	0.09	0.82	11	Present study (9/98) (47°08', 122°54')
Totten Inlet						
	Taylor United Shellfish (c)	1.00	0.00	0.00	32	Present study (7/97) (48°13', 123°06')
	Taylor United Shellfish (n)	0.75	0.25	0.00	4	Present study (7/97)
	Taylor United Shellfish	1.00	—	0.00	58	Anderson et al. 2002
	Carlyon	0.08	—	0.92	26	Anderson et al. 2002
Hammersley Inlet						
	Shelton Yacht Club*	0.43	0.57	0.50	14	Present Study (9/98) (47°13', 122°05')
	Shelton	0.33	—	0.67	26	Anderson et al. 2002
	Kamilche Sea Farms	0.00	0.19	0.81	63	Brooks 1991
Case Inlet						
	Grapeview Marina	0.09	—	0.91	32	Anderson et al. 2002
	Joemma Beach State Park	0.02	—	0.98	58	Anderson et al. 2002
Carr Inlet						
	Penrose Point State Park	0.02	—	0.98	60	Anderson et al. 2002
	Purdy	0.04	—	0.96	52	Anderson et al. 2002
Strait of Juan de Fuca						
	Vancouver Island					
	Sooke Harbour	0.00	0.01	0.99	73	Heath et al. 1995
	Victoria	0.00	0.03	0.97	30	Heath et al. 1995
Sequim						
	John Wayne Marina	0.00	1.00	0.00	9	Brooks 1991
		0.00	0.18	0.82	11	Present study (10/98) (48°04', 123°06')
	Washington Harbor	0.00	0.00	1.00	63	Brooks 1991
	Van Riper's Marina, Sekiu	0.10	0.00	0.90	10	Present study (10/98) (48°16', 124°18')
	Makah Marina, Neah Bay	0.00	0.08	0.92	13	Present study (10/98) (48°22', 124°37')
	Port of Port Angeles	0.23	0.31	0.46	13	Present study (10/98) (48°07', 123°26')
	Tatoosh Island, Cape Flattery	0.17	0.00	0.83	6	Suchanek et al. 1997
		0.00	0.00	1.00	92	Present study (6/00) (48°23', 124°44')
Washington outer coast						
	Ruby Beach	0.00	0.00	1.00	72	Brooks 1991
	Westport, Grays Harbor	0.00	0.00	1.00	63	Brooks 1991
Willapa Bay						
	Bay Center	0.00	0.00	1.00	81	Brooks 1991
	Port of Willapa	0.00	0.00	1.00	54	Brooks 1991
Columbia River		0.00	0.00	1.00	54	Brooks 1991
Oregon coast						
	Tillamook Bay	0.00	0.00	1.00	25	McDonald and Koehn 1988 ^c
		0.00	0.00	1.00	121	McDonald and Siebenaller 1989
		0.00	0.00	1.00	17	Suchanek et al. 1997

TABLE 1.
continued

Location	Site	Mg	Mg × Mt	Mt	N	Source
Yaquina Bay	Yaquina Bay	0.00	0.11	0.89	54	Brooks 1991
		0.00	0.00	1.00	338	McDonald and Siebenaller 1989
	Newport	0.00	0.00	1.00	25	McDonald and Koehn 1988
		0.00	0.00	1.00	68	Rawson and Hilbish 1995
		0.00	0.00	1.00	144	McDonald and Siebenaller 1989
Alsea Bay	Umpqua River	0.00	0.00	1.00	111	McDonald and Siebenaller 1989
	Coos Bay	0.09	0.00	0.91	43	Suchanek et al. 1997
	Port Orford	0.00	0.00	1.00	25	McDonald and Koehn 1988
		0.00	0.00	1.00	30	Rawson and Hilbish 1995
California coast	Crescent City	0.00	p?	p	21	McDonald and Koehn 1988
		0.06	0.00	0.94	84	Rawson et al. 1999
		p	nd	nd	≥32	Sarver and Loudenslager 1991
		0.04	0.04	0.92	48	Sarver and Foltz 1993
		0.00	0.00	1.00	29	Rawson and Hilbish 1995
Humboldt Bay	Humboldt Bay	0.88	0.08	0.04	98	Brooks 1991
	Humboldt Bay	p	nd	nd	≥59	Sarver and Loudenslager 1991
		0.00	0.00	1.00	34	Rawson and Hilbish 1995
	Arcata Bay	0.01	0.01	0.98	83	Rawson et al. 1999
		0.00	0.00	1.00	25	McDonald and Koehn 1988
	Eureka Slough	0.00	0.01	0.99	192	Sarver and Foltz 1993
	Woodley Island	0.00	0.00	1.00	60	Sarver and Foltz 1993

Notes:

^a ITS alleles only. This marker did not distinguish *M. galloprovincialis* from the introduced Atlantic *M. edulis*, so samples listed here under *M. galloprovincialis* may have included both.

^b Mussels in present study selected for large size (all 3–10 cm shell length).

^c Mussels in Brooks (1991) selected for *M. galloprovincialis*-type morphologies.

^d In Anderson et al. (2002), hybrids were not distinguished from individuals with only *M. galloprovincialis* alleles.

^e *Mpi* alleles only.

been reported from Washington (Suchanek et al. 1997), and further sampling is warranted to determine whether *M. galloprovincialis* is invading intertidal as well as subtidal habitats.

Oregon and Northern California

On the Oregon coast, *M. galloprovincialis* alleles have been reported only from Yaquina and Coos Bays (see Table 1). Surveys in northern California (north of Cape Mendocino) repeatedly identify *M. galloprovincialis* alleles in Crescent City and Humboldt Bay (see Table 1). The central and southern California distribution of *M. galloprovincialis* is summarized elsewhere (McDonald & Koehn 1988, McDonald et al. 1991, Sarver & Foltz 1993, Suchanek et al. 1997).

Allele Frequencies

For a subset of sites in three Northeast Pacific regions (those sites at which *M. galloprovincialis* alleles were reported, and genetic markers distinguished heterozygotes from homozygotes), mean genotype frequencies were calculated (Table 2). In each region the observed frequencies differed significantly from Hardy-Weinberg equilibrium in goodness-of-fit tests using the total number of each genotype (see Table 2). These departures from equilibrium reflect an under-representation of *M. galloprovincialis* alleles, which is consistent both with the early stages of an invader's spread into a native population and with more general observations

of heterozygote deficiency in *Mytilus* populations (Raymond et al. 1997).

Invasion Pathways

It seems likely that *M. galloprovincialis* has been introduced to the Northeast Pacific through both aquaculture and shipping or boating. Most commercial *M. galloprovincialis* seed in the region is currently supplied by a single farm in Washington, which originally obtained its stock from California in the 1980s (G. King, Taylor United Shellfish, pers. comm.). These mussels were initially imported because they seemed to be resistant to bivalve disseminated-hemic neoplasia, a disease of unknown pathogenic agent that affects native *M. trossulus* particularly in culture (Brooks & Elston 1989a, Brooks & Elston 1989b). At the time of import, the genetic identity of the disease-resistant Californian mussels was not recognized (G. King, pers. comm.). The introduction date of *M. galloprovincialis* to California is unknown, but it has been suggested that historical records of a *Mytilus* invasion in southern California (Burch 1943–1958, Smith 1944, Coe 1945, 1946) reflect the arrival of *M. galloprovincialis* (Carlton 1979, Geller 1999). Aquaculture farms currently serve as localized sources of reproductively mature *M. galloprovincialis* whose larvae presumably disperse as far as currents permit. Additional dispersal within the region may occur from wild populations and via adult mussels transported on boat hulls.

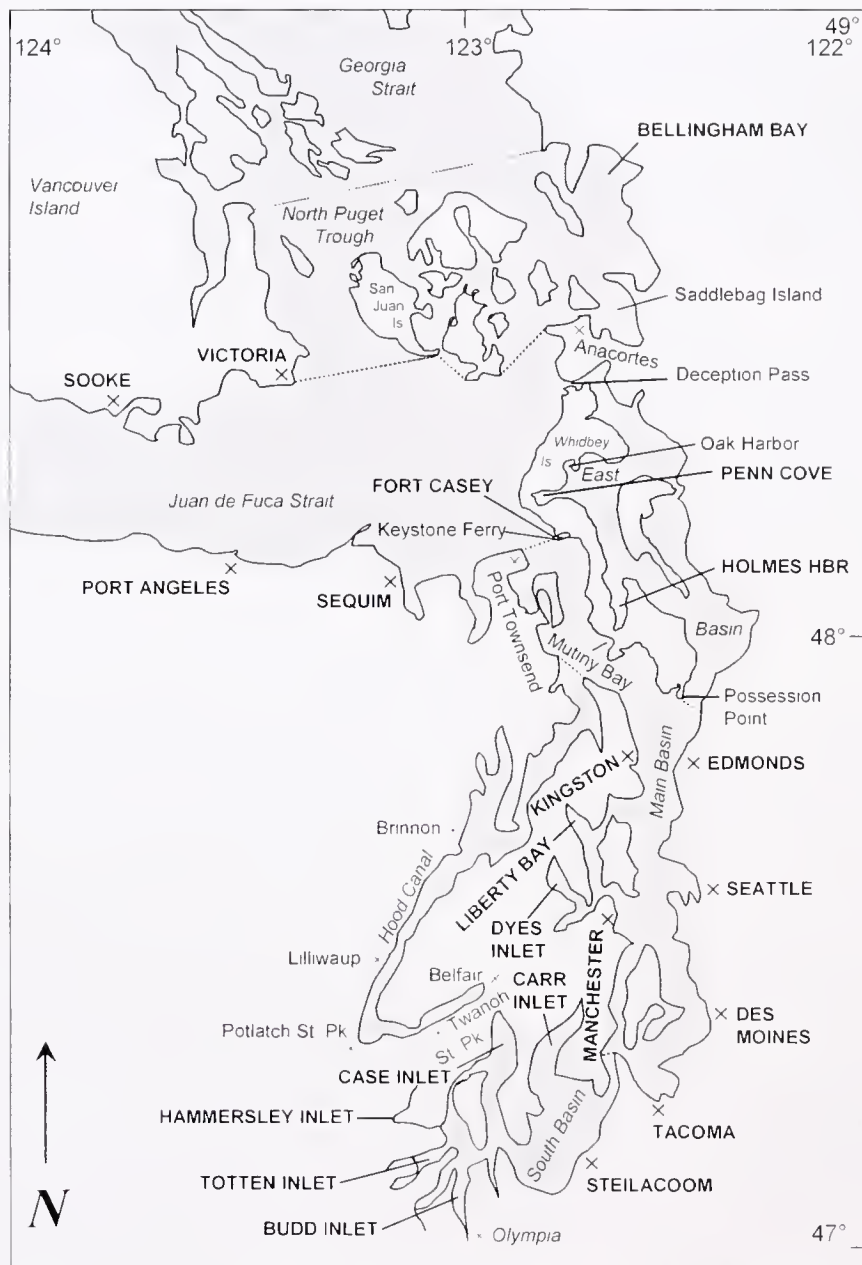


Figure 2. *Mytilus* spp. sampling sites in Puget Sound and Puget Trough, Washington. Sites where *M. galloprovincialis* genes were detected indicated in bold capitals. Sites where only *M. trossulus* genes were detected indicated in regular type. Landmarks indicated in italics. For details see Table 1.

Ship ballast water and ship and boat hulls may also provide a continual supply of organisms, including *M. galloprovincialis*, to Pacific Northwest waters (Carlton & Geller 1993, Geller et al. 1994, Suchanek et al. 1997). Shipping to the region comes primarily from Asia, where *M. galloprovincialis* is also introduced (Wilkins et al. 1983, Lee & Morton 1985, Inoue et al. 1995, Inoue et al. 1997, Suchanek et al. 1997, Wonham & Carlton 2004). This pathway may be particularly relevant to *M. galloprovincialis* populations along the Strait of Juan de Fuca, where Vancouver-bound vessels may deballast (Larson et al. 2003, Levings et al. 2004).

Within the northeast Pacific, no study to date has explicitly been designed to assess the spatial spread of mussels from aquaculture facilities, ports, or marinas. Detailed genetic investigation of possible source populations (i.e., Japan, California, and the

Mediterranean) may shed light on the likeliest invasion pathways for *M. galloprovincialis* to the region (Rawson & Hilbish 1995a, Sanjuan et al. 1997, Quesada et al. 1998). Within the region, sampling a range of mussel sizes and habitats (i.e., intertidal and subtidal; rock and floats) at increasing distances from farms, ports, and marinas could provide a clearer picture of the role of these potential *M. galloprovincialis* sources. Because the species is now widespread, more ecologically interesting questions concern its success and impacts as an invader (e.g., Hockey & Van Erkom Schurink 1992, Geller 1999, Gilg & Hilbish 2000, Secor et al. 2001).

The combined results reported here identify a major zone of sympatry and hybridization between *M. galloprovincialis* and *M. trossulus* in Washington waters, in addition to the well-recognized

TABLE 2.

Mean (± 1 SD) for homozygote and heterozygote *Mytilus* allele frequencies in 3 regions of the northeast Pacific from Table 1 (number of sites per region in parentheses). Allele frequencies in all 3 regions for Mg, homozygous *M. galloprovincialis*, Mt, homozygous *M. trossulus*, and Mg \times Mt, heterozygous hybrids, are significantly different from Hardy-Weinberg equilibrium at $p < 0.0001$, based on contingency table tests using the number of mussels of each genotype.

Site	Mg	Mg \times Mt	Mt	χ^2
Puget Sound (11)	0.18 (0.23)	0.24 (0.20)	0.63 (0.30)	228.9
Strait of Juan de Fuca (8)	0.06 (0.09)	0.20 (0.34)	0.74 (0.34)	136.5
Oregon & California (7)	0.15 (0.32)	0.04 (0.04)	0.81 (0.34)	495.3

California zone (McDonald & Koehn 1988, McDonald et al. 1991, Sarver & Foltz 1993, Suchanek et al. 1997). The apparent absence of *M. galloprovincialis* genes between Yaquina Bay, Oregon, and Cape Flattery, Washington, may reflect the absence of mussel culture and major shipping ports along this stretch of coastline. On

the other hand, given coastal currents and shipping traffic (Levings et al. 1998, Larson et al. 2003), and the associated potential for larval release and dispersal, it would not be surprising if further sampling revealed that the Washington and California zones comprised a larger *Mytilus*-complex hybrid swarm extending along the North American Pacific coast.

ACKNOWLEDGMENTS

Many thanks to J. Mitton and B. Kreiser at the University of Colorado, Boulder, and S. Edwards at the University of Washington, Seattle, for making laboratory space, equipment, and reference samples available. K. Ward provided expert laboratory assistance, and K. Brooks and two anonymous reviewers provided valuable comments. G. King and Taylor Shellfish Inc. generously made mussel samples available. This work was supported by Graduate Research Fellowship NA77OR0250, Estuarine Reserves Division, Office of Ocean and Coastal Management, National Ocean Service, National Oceanic and Atmospheric Administration (Padilla Bay National Estuarine Research Reserve). Access to Tatoosh Island was permitted by the Makah Tribal Council.

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VARIATIONS IN DENSITY, SHELL-SIZE AND GROWTH WITH SHORE HEIGHT AND WAVE EXPOSURE OF THE ROCKY INTERTIDAL SNAIL, *CALYPTRAEA SPIRATA* (FORBES, 1852), IN THE TROPICAL MEXICAN PACIFIC

EDUARDO RÍOS-JARA, CINTHYA CAROLINA HERNÁNDEZ CEDILLO,
EDUARDO JUÁREZ CARRILLO AND ILDEFONSO ENCISO PADILLA

Laboratorio de Ecosistemas Marinos y Acuicultura, Departamento de Ecología, Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Apartado Postal 52-114, Zapopan, Jalisco 45035 México.

ABSTRACT This study evaluates the density, shell-size, and growth of the intertidal snail, *Calyptrea spirata* (Forbes 1852), in the beach of Teopa of Bahía Chamela, Jalisco, located in the tropical Mexican Pacific. The snail is one of the most important resources from the rocky shores of the region. Gastropod density and the size of the shell (maximum diameter and height) were estimated in the upper and lower intertidal zones and in wave-exposed and wave-protected areas of the beach on December 1999. Results showed that density is lower in the upper intertidal zone and increases in the lower intertidal zone (ANOVA, $P < 0.05$). The species was not registered in the supralittoral zone, while in the infralittoral zone it was observed to a depth of 3 m. There was no difference in the size of the shell between snails from the upper and lower intertidal zones (ANOVA, $P = 0.30$), although bigger shells (maximum diameter = 5.0 cm) were more frequently observed in the lower zone. Shell diameter was bigger in the exposed than in the sheltered areas of the beach (ANOVA, $P < 0.05$). The growth rate of snails smaller than 3 cm was higher (0.268 cm/month) than in bigger snails (0.077 cm/month) (ANOVA, $P = 0.037$). The ANCOVA test indicated that the least squares regressions of maximum diameter versus height of the shell correspond to differentiated morphologic responses to the wave intensity effect but no difference in these associations was detected in response to shore height.

KEY WORDS: density, shell-size, *Calyptrea spirata*, growth, Mexican Pacific

INTRODUCTION

It has been shown that wave intensity plays a major role in determining the abundance and vertical distribution of organisms living in the intertidal zone (Lewis 1964). In the Pacific shores, wave action intensity is particularly important because the extensive continuous surface area of this ocean makes longer and more intense waves relative to other oceans of the world (Ricketts et al. 1997). The rocky intertidal environment of the tropical Mexican Pacific includes a great variety of mollusks, gastropods being the most abundant and diverse. Many high energy rocky shores of this region are characterized by a dynamic topographic structure with a steep environmental gradient over short distances across the intertidal zone. Thus, the tidal regimen determines temperature, desiccation, and submersion time gradients, those that influence the organisms that inhabit intertidal shores (Levinton 1984). All these factors together with the biologic factors such as density or predation define the abundance and the zonation patterns of gastropod populations.

Differences in the size of the shell and growth of gastropods have been related to several physical conditions of the habitat including wave intensity (Brown & Quinn 1988, Denny 1994, De Wolf et al. 1999, Giraldo-Lopez & Gómez-Schouben 1999) and shore height (Vermeij 1972, Hobday 1995, Giraldo et al. 2002, Tanaka et al. 2002). Furthermore, density is considered responsible for intraspecific differences in shell size (Giraldo et al. 2002) and individual growth rate variability of gastropods (Haven 1973, Black 1977, Underwood 1978, Williamson and Kendall 1981).

The growth of mollusks have been traditionally estimated through the use of direct methods based on measurements of the size of the shell of individuals at different intervals of time (Gómez-Marquez 1994). The mark-recapture method is reliable to

estimate mollusk growth (Hughes & Roberts 1980, Phillips 1981, Fletcher 1984, Chow 1987, Katoh 1989, Bowling 1994, Takada 1995, Iijima 2001). In the Mexican Pacific, differences in the growth rate of the wavy turban snail, *Astrea undosa*, have been related to wave intensity (Cupul-Magaña & Torres-Moye 1996, Gluyas-Millán et al. 1999). Other studies describe variations in the size of the shell among populations living in areas with different wave exposition. In populations of the rocky intertidal snail *Thais* sp. from Baja California, the shell is shorter and heavier in exposed beaches and longer and slender in protected beaches (Kitching 1976). Similarly, the shell of the limpet *Siphonaria gigas* is wider when living in more wave exposed rocky beaches (Giraldo-López & Gómez-Schouben 1999), and the size of the chiton *Katharina tunicata* from northern California may be smaller in areas with stronger wave action (Stebbins 1988).

In the coast of Jalisco, México, there are few studies on growth and shell size of snails. Probably, the purple snail *Plicopurpura pansa* is the only species studied so far (Ríos-Jara et al. 1994, Michel-Morfin et al. 2002). One of the most important potential resources from the rocky shores of Jalisco is the so-called "gorrito" (small cap) snail *Calyptrea spirata* (Forbes 1852). The snail is easily distinguished for its conical limpet-like shell with large, coarsely ribbed, whitish or grayish outside, dark brown within without operculum. Its distribution ranges from Mazatlán to the Gulf of Tehuantepec, México (Keen 1971) and the Gulf of California (Morris 1966). In the coast of Jalisco, the species has been registered in the beaches of El Tamarindo, La Calechosa, Los Angeles (González-Villarreal 1977); Yelapa (Fonseca Madrigal 1998); Bahía Tenacatita (Hernández 1998), and in Bahía Cuastecomate (Esqueda et al. 2000). The muscular foot of *C. spirata* is most esteemed by fishermen who used it for food. The species has also a cultural traditional value in the region. It was used as a burial offering in pre Hispanic funerals recovered in the archaeological site of Salagua, Colima, dated between 900–1530 years B.C.

(Fieldman 1968). Recently, local populations have been diminished and in some beaches the species has disappeared, probably because of the intense fishing and deficient management activities. Although the taxonomic composition and abundance of rocky intertidal mollusk communities from the coast of Jalisco is well known, only a few studies focus on certain species, particularly on the population ecology of *Plicopurpura patula pansa*, a commercially important rocky intertidal snail (see Ríos-Jara et al. 1994, 2001, Pérez Peña & Ríos-Jara 1998, Esqueda et al. 2000 for a list of this research).

There are no previous studies on the population ecology of *C. spirata*. Therefore, the major objective of this study is to evaluate the abundance, vertical distribution, size, and growth of this species in Bahía Chamela, Jalisco, in the tropical Mexican Pacific. The study examines a population located in the beach of Teopa, in the southern part of the bay, which is well represented in terms of abundance, distribution, and size of the organisms. The size of the shell and the rate of growth were estimated in the upper and lower intertidal levels and at two different wave exposition conditions. This study provides basic knowledge concerning the factors that affect the growth and development of the species, which may be used to suggest future recommendations needed for the sustainable harvesting of the snail.

MATERIALS AND METHODS

Study Site

Chamela Bay is located in the central portion of Jalisco, México. The bay spreads 23.3 by 3 km between Punta Rivas and Punta Farallón (19°34'50"N to 19°23'20"N, 105°07'30"W to 105°01'40"W) and has a surface area of approximately 6,690 hectares. The region has warm-wet climate with the rainy season occurring mostly during the summer. Annual temperature ranges from 32.3°C in September to 20.6°C in January (mean = 25.2°C). Cumulative monthly precipitation ranges between 587.5 and 967.3 mm. September records the highest precipitation values with 301.7 mm and February the lowest with 1.6 mm (García 1973). There is a mixed tidal cycle with two unequal high and two unequal low tides each day.

The shoreline of the bay is composed of sandy and rocky beaches. The rocky beaches usually have solid blocks sometimes mixed with fixed or loose boulders and pebbles. The rocky beach of Teopa (19°23'33"N, 105°01'24"W) is located in the southern portion of the bay. The topography of the beach is very irregular with steep and smooth surfaces and heterogeneous rock platforms with water retaining cracks, crevices, and tidal pools. This irregular profile (slope = 60° to 90°) has many habitats with different degrees of exposure to wave action from sheltered to very exposed conditions through the intertidal zone. This beach was chosen because the population of *Calyptraea spirata* is well represented in terms of abundance, distribution, and size of the organisms. Preliminary observations were necessary to characterize the zonation of conspicuous species in the intertidal where *C. spirata* lives. Intertidal levels were determined using sessile forms, particularly macroalgae, crustaceans, and mollusks.

Methods

The beach of Teopa has a narrow intertidal zone (3–5 m). Two different levels were determined (upper and lower intertidal) for the study of gastropod density, shell size, and growth. At the same

time, 2 areas of the beach with different degrees of exposure to wave action were chosen: (1) a more sheltered part of the beach with relatively less wave intensity, and (2) a very exposed surf sweeping area with strong wave action. Sample size was estimated previously according to the cumulative means technique (Elliott 1977). For this technique, the cumulative mean density of snails was plotted as a function of the cumulative number of replicate samples or the cumulative area sampled. As the number of samples increased, the fluctuation of the mean decreased. The number of replicates was considered sufficiently large when such fluctuations were so slight that the cumulative mean became insensitive to variations in the data.

Density and size structure of the population found within the upper and lower intertidal zones and two wave exposure conditions (exposed and protected) were estimated during low tides in December 1999. The plot method was used for density estimations (Brower & Zar 1977). Line transects of 30 m and square plots (quadrats) of 0.25 m² were used. The procedure involved placing a series of 30 quadrats along each transect line, 15 on each side. The line was marked and numbered every 50 cm (60 numbers on each side) and a table of random numbers was used to choose the position of the quadrats along the transect. The number and size of snails found within each quadrat were estimated. The optimal sample size (25–30 quadrats) suggested by the cumulative means technique was used. A two-way ANOVA ($P < 0.05$) was used to determine whether the mean density (ind./m²) differed between intertidal zones and among the two wave intensity conditions. A posthoc test (Dunnett) was used to contrast mean densities estimated in the upper and lower intertidal zones and the protected and exposed areas.

The snails were measured to nearest 0.1 mm with a hand vernier caliper (maximum diameter and height of the shell). Maximum diameter values were used to make histograms of the size frequency distributions. Bimodality was tested by applying a 5% level of significance hypothesis test (Reschenhofer 2001). Differences in the size of the shell related to tidal level and wave exposure conditions were tested using a two-way ANOVA ($P < 0.05$). The normality and the homocedasticity of data (Bartlett's test) was examined, and the logarithmic transformation ($\log X + 1$) was performed when necessary (Zar 1999). If these conditions were not fulfilled, the non parametric mean comparison test of Kruskal-Wallis was used. The relationship between maximum diameter and height of the shell of individuals from different tidal levels and wave exposure conditions was described with least-squared regressions (Statistica Program SYNTAX 0.5 for Windows). The resulting equations were contrasted using an ANCOVA analysis (SPSS and SigmaStat Programs).

The growth rate was estimated during monthly samplings from October 1999 to May 2000 at the same areas and intertidal levels of the beach of Teopa where gastropod density and shell-size were studied. During the first sampling event, the shells of 300 snails from the upper and lower intertidal levels were measured (maximum diameter of the shell). Each individual was marked using plasticized epoxy glue (Plastiloca) and labels with printed progressive numbers. The labels were then coated with transparent instant nail varnish to preserve the numbers. All snails were released to their original position. The following months, marked individuals were measured and the numbered labels renewed when necessary. Increments in the size of the shell were documented and expressed in cm/week. New shells were labeled when the number of marked individuals decreased, so the total number was always similar to

the original. Sometimes, the somehow lost or not found snails were found again in later samplings. Individual growth rates (G) of the recaptured snails were estimated using the equation (Iijima 2001):

$$G = \Delta X / X (30/T)$$

where T is the time (month) from the last release to recapture, X the maximum diameter of the shell at the last release, ΔX the increment in maximum diameter during the period T .

For comparative purposes, snails were divided in 2 groups: Group 1, included snails smaller than 3 cm of maximum diameter and Group 2, those snails of more than 3 cm of maximum diameter. A one-way ANOVA ($P < 0.05$) was performed to compare the growth rate of both groups of snails.

RESULTS

Mean density of *Calyptraea spirata*, increases from the upper to the lower level of the intertidal zone (Fig. 1). The density was lower in the upper intertidal zone compared with the lower intertidal zone of both the protected and the exposed areas (ANOVA, Dunnett test, $P < 0.05$). The species was not registered in the supralittoral zone in any area of the beach, whereas in the infralittoral zone it was observed (but not sampled for density estimations) to a depth of 3 m. Snails in the infralittoral were also attached to rocky substrata, defining a wide vertical distribution from the upper intertidal to the shallow infralittoral zones. Within the same shore height (upper or lower intertidal), no significant variation in gastropod density was detected between the sheltered and the exposed parts of the beach (ANOVA, $P > 0.05$).

Histograms of the size frequency distributions of individuals show at least one mode in upper intertidal zone of both the protected and the exposed areas of the beach (Fig. 2). In the protected area, the mode extends along all size ranges of the histogram, from 0.4–5.0 cm: in the exposed area, the mode includes the ranges of 1.0–4.5 cm. According to the hypothesis test ($P < 0.05$), histograms of individuals from the lower intertidal are bimodal. In the protected area, the modes occur in the size ranges of 1.0–1.5 and 3.1–3.5 cm of shell diameter; in the exposed area, the modes occur in the size ranges of 1.6–2.0 and 3.1–3.5 cm. During the study, the diameter of the shell ranged from 0.40 cm for the smallest individual to 5.0 cm for the largest individual measured. The mean diameter was found to be 2.86 ± 0.75 mm (\pm one standard deviation).

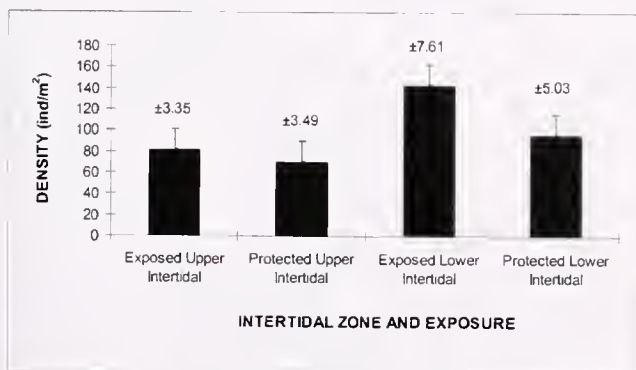


Figure 1. Mean density (ind/m² \pm one standard deviation) of *Calyptraea spirata* in the beach of Teopa, Jalisco (December, 1999). Estimated densities of two areas of the beach with different degrees of exposure to wave intensity (exposed and protected) at two intertidal zones are shown.

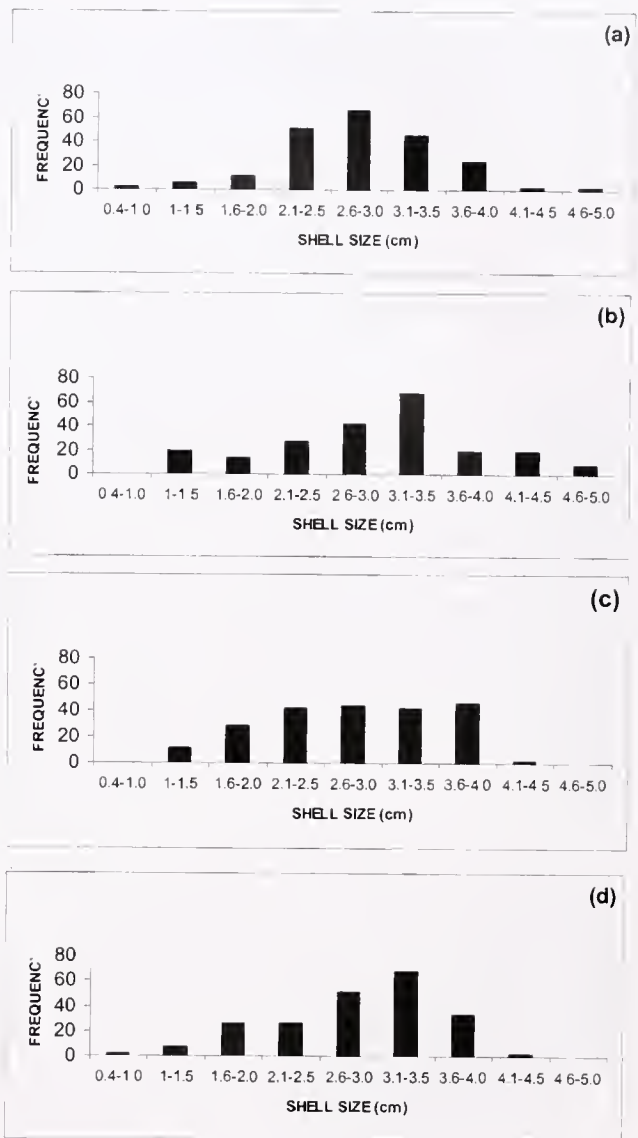


Figure 2. Histograms of the size frequency distributions of individuals at two intertidal zones with different degrees of exposure to wave intensity in the beach of Teopa, Jalisco (December, 1999). (a) Protected Upper intertidal; (b) Protected Lower intertidal; (c) Exposed Upper intertidal; (d) Exposed Lower intertidal.

tion). The most common repeated value of diameter was 3.1 cm (by 3.5%), 3.0 cm (by 2.8%), and 2.8 cm (by 2.5%).

The homoscedasticity condition was not fulfilled by shell diameter even with logarithmic transformation (Bartlett test, $P < 0.01$), so the non parametric test of Kruskal-Wallis was used to detect differences in shell size. Although the biggest individuals were always found in the lower intertidal zone, no significant difference in shell diameter between snails from the upper and lower intertidal zones was detected (ANOVA, $P > 0.30$) (Table 1). However, the shell-diameter of *C. spirata* was bigger at the most wave-exposed areas compared with the wave-protected areas of the beach (ANOVA, $P < 0.05$).

Individual growth rates did not varied greatly between individuals from different shore heights and wave exposure conditions (Table 2). The growth rates were significantly different in only 2

TABLE 1.

Mean size of the shell (maximum diameter and height, cm \pm one standard deviation) of *Calyptraea spirata* at 2 intertidal zones of the beach of Teopa, Jalisco with different degrees of exposure to wave intensity (exposed and protected) (December, 1999).

Intertidal Zone		n	Exposed	n	Protected	Mean
Upper	Maximum diameter	216	2.79 \pm 0.74	212	2.83 \pm 0.67	2.81 \pm 0.70
	Height	216	1.85 \pm 0.57	212	2.02 \pm 0.47	1.93 \pm 0.53
Lower	Maximum diameter	218	2.85 \pm 0.70	220	2.97 \pm 0.87	2.91 \pm 0.79
	Height	218	1.85 \pm 0.51	220	1.92 \pm 0.68	1.91 \pm 0.60
Mean	Maximum diameter	434	2.82 \pm 0.72	432	2.90 \pm 0.78	2.86 \pm 0.75
	Height	434	1.85 \pm 0.54	432	1.96 \pm 0.59	1.91 \pm 0.57

situations: (1) when comparing all snails of group 1 versus group 2, the growth rate of individuals smaller than 3 cm (Group 1) was significantly higher than the growth rate of larger individuals (Group 2) (ANOVA, $P = 0.037$); and (2) in the case of snails of group 2 inhabiting the upper intertidal, results indicated that there is a significant difference in the growth rate between exposed and protected conditions (ANOVA, $P = 0.018$).

The least squares regressions of maximum diameter versus height of the shell were examined by shore height and by wave exposure conditions (Fig. 3). All associations were significant ($P < 0.05$). The ANCOVA test indicated that these associations correspond to differentiated morphologic responses to the wave intensity effect ($P < 0.05$), whereas regressions calculated in the two different intertidal zones were not significantly different ($P > 0.05$).

DISCUSSION

The intertidal area of Teopa beach shows important environmental differences that explain the vertical gastropod variation found in this study. Several biotic factors should have an important role in the explanation of the observed density, shell size, and growth rate variations; including the human impact on gastropod populations; and the natural predation and competition.

The harvesting pressure by humans was considered responsible for the small size of *Monodonta labio*, an edible rocky intertidal

snail in Amakusa, Japan (Iijima 2001). Furthermore, the growth rate in Amakusa was slower than in other localities possibly because the selective collection of large *M. labio* by human. In Chamele Bay, fishermen use different mollusk species (limpets, snails, mussels, chitons and octopuses) from the rocky shores. *Calyptraea spirata* is preferred over all other intertidal and shallow subtidal gastropods. The last report of the capture of the snail from the government's Fishery Office in Puerto Vallarta, Jalisco, indicate a very small volume of 106 Kg in 1995 (Esquivel & Plascencia 1999). In recent years, the capture of the snail is no longer reported by the Mexican government, probably because of a decrease of the previously exploited populations and the absence of adequate mechanisms to obtain accurate information on the harvesting of the snail. Nowadays, the species is found abundantly only in a few beaches of Jalisco that may be considered as relics, because access is difficult for fishermen. Although access to Teopa beach is restricted mainly because the entrance walkways go across private properties, the beach is regularly visited by local people. The snail is mostly harvested from the upper intertidal zone where it is easier to reach and there are less hazardous conditions. The strong waves and heavy surf of the lower intertidal prevent fishermen from collecting the snails even in the sheltered areas of the beach. On the other hand, natural predation also affects gastropod density and shell-size in tropical intertidal habitats (Palmer 1979, Garrity & Levings 1981, Menge & Lubchenco 1981). Shell-breaking preda-

TABLE 2.

Growth rate (cm/week) of *Calyptraea spirata* from different tidal levels and wave exposure conditions.

Tidal Level and Wave Exposure		Group 1 Shells <3 cm		Group 2 Shells >3 cm		
		Growth Rate (cm/month)		Growth Rate (cm/month)		
Upper intertidal		Exposed	Protected	Exposed	Protected	
		0.180	0.312	0.040	0.028	
Lower intertidal		(n = 42)	(n = 69)	(n = 48)	(n = 26)	
		0.360	0.220	0.080	0.160	
Total individuals of each group		(n = 30)	(n = 27)	(n = 27)	(n = 39)	
		0.268		0.077		
	<i>n</i>	ANOVA Test	<i>P</i> Value	<i>n</i>	ANOVA Test	<i>P</i> Value
Upper intertidal	111	Exposed vs. Protected	0.155	74	Exposed vs. Protected	0.018*
Lower intertidal	57	Exposed vs. Protected	0.747	66	Exposed vs. Protected	0.812
Exposed	72	Upper zone vs. Lower zone	0.543	75	Upper zone vs. Lower zone	0.942
Protected	96	Upper zone vs. Lower zone	0.723	65	Upper zone vs. Lower zone	0.124
Total individuals of each group	308	Group 1 vs. Group 2				0.037*

* Significant difference in individual growth rate, ANOVA, $P < 0.05$.

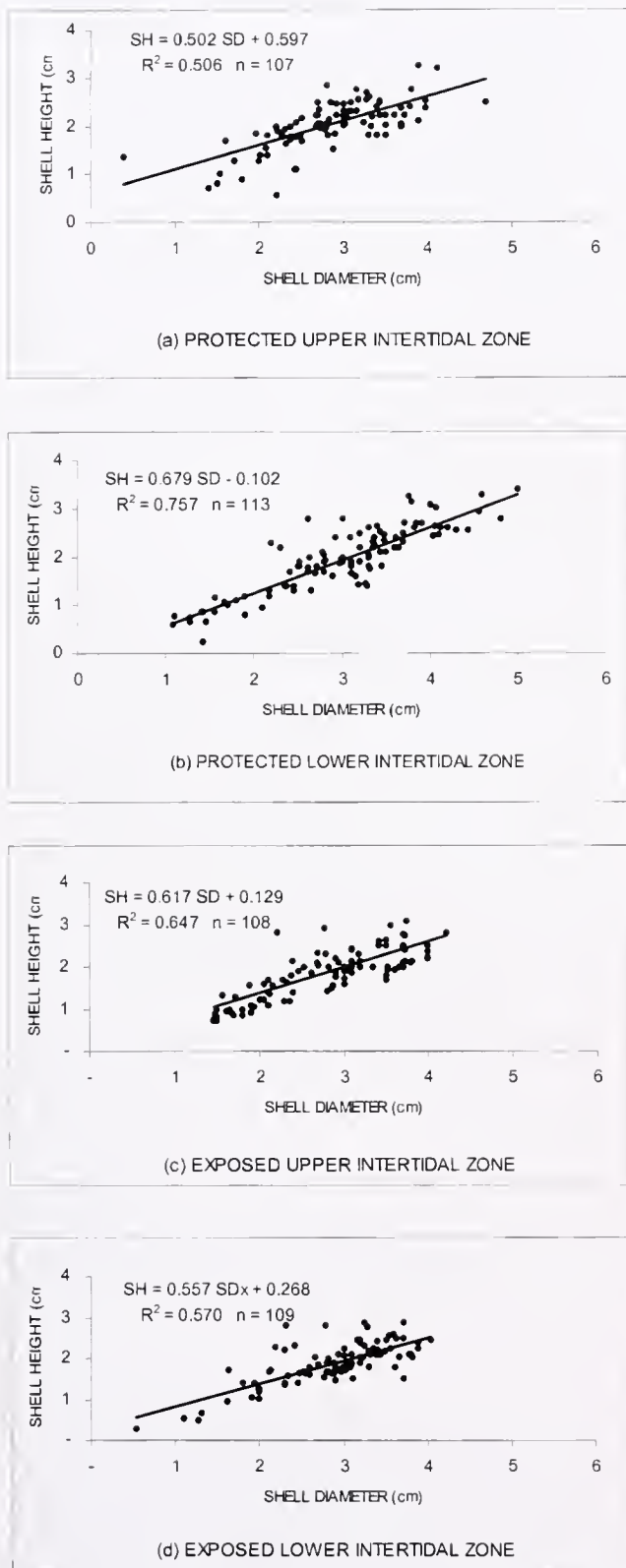


Figure 3. Fitted linear regressions of shell diameter (SD) vs shell height (SH) for *Calyptraea spirata* at two intertidal zones with different degrees of exposure to wave intensity in the beach of Teopa, Jalisco (December, 1999). (a) Protected Upper intertidal; (b) Protected lower intertidal; (c) Exposed Upper intertidal; (d) Exposed Lower intertidal.

tors of gastropods include fishes and crustaceans (Vermeij 1978, Bertness & Cunningham 1981). In the rocky shores of Jalisco, several mammals and sea birds have been observed as predators of snails (Ríos-Jara et al. 1994). During the high tide periods, predation by fishes and crustaceans may be more intense while during the low tide periods, predation by mammals and birds is more important. Consequently, the predation intensity should be influenced by intertidal shore height and it is probably related to the vertical variation in density and shell-size found for *C. spirata* in Teopa beach.

The interspecific and intraspecific competition for food has a considerable effect on gastropod density (Underwood 1979, Creese & Underwood 1982, Underwood & Denley 1984) and growth rates (Paine 1969) in rocky intertidal habitats. Food resources have been also considered as responsible for differences in growth rates in intertidal gastropods (Takada 1995). The members of the Family Calyptraeidae are filter-feeding gastropods that consume microplankton and suspended organic matter from water (Ruppert & Barnes 1996). Field observations indicate that *C. spirata* may have this feeding strategy, because snails were seen rising the shell from the substratum only during the upcoming and lowering tides. Little or no movements of marked individuals were detected during the study, indicating that snails do not need to move much to filter food items from the surrounding waters. Therefore, gastropod density difference through the intertidal zones may be related to the tidal regimen, which determines an emergence and submersion time gradient, and influence the food availability for gastropods.

Previous studies have suggested that the density of intertidal gastropods is affected by abiotic factors such as wave action, temperature, and desiccation (Atkinson & Newbury 1984, Raffaelli & Hawkins 1996, Gluyas-Millán et al. 1999, Tanaka et al. 2002). Differences in shell-size and growth rate have been explained in response to the effects of wave exposure and shore height (Crothers 1992, Giraldo et al. 2002). In the case of *C. spirata*, the growth rate of individuals smaller than 3 cm was significantly higher than the growth rate of larger individuals. Similarly, Williamson and Kendall (1981) found that the growth rate of the rocky intertidal snail *Monodonta lineata* from the coast of England decreases with the size of the individuals. The same situation was later found for *M. labio* in Japan (Iijima 2001). Differences in the growth rate between smaller and bigger individuals may be related to the reproductive effort of adults. Thus, sexual maturity may imply more energy used for reproduction instead of shell growth.

The tropical intertidal zone experiences continuous severe physical conditions during daytime exposures to sun and air. Substratum temperatures in tropical rocky shores may be extreme in sunny weather, and lethal stress levels can be reached on open surfaces on a clear day (Giraldo et al. 2002). Although, *C. spirata* live on the exposed areas of the rocks where the wave action and the sunlight are important restrictive factors (Santés-Alvarez & Hernández 1983, Williams & Morritt 1995). Adaptations to the exposed areas of *C. spirata* include the wide cap-shaped hydrodynamic shells that diminish the effect of direct wave action and tidal currents; a wide shell aperture, and an extensive foot that gives a stronger attachment to the rocky substratum. The rigor of desiccation and extreme temperatures in the rocky intertidal increases according to a gradient from low to high shore heights. Therefore, those gastropod species living through the intertidal zone may have density variations related to this gradient. *Calyp-*

traea spirata has a wide vertical distribution, but the number of snails increases from the upper to the lower level of the intertidal zone. Other studies have shown variable vertical distribution patterns in the rocky beaches of Jalisco; many gastropod species also increase their numbers from the supralittoral to the lower tidal zones whereas the opposite occurs in other species (Esqueda et al. 2000, Ríos-Jara et al. 2001). Marked differences in vertical distribution of species or their absence in some intertidal levels closely relate to the transient nature of the littoral environment, which changes from nearly terrestrial to completely marine conditions. Environmental cues that govern distribution of these species were not evaluated, but we predict that they respond to features of the habitat, which are very variable across the shore.

Several other factors (e.g., mating, feeding, and interspecific relationships) may be important regulating factors. Vertical distribution of many gastropod species closely relate to the presence of other invertebrates in the rocky beaches of California (Sledner 1981) and Baja California (Ríos-Jara 1983). Other species are similarly associated with the mats of macroalgae and the aggregations of mussels (*Brachidontes* sp. and *Chloromytilus* sp.) in the coast of Jalisco (Esqueda et al. 2000). *Calyptrea spirata* form aggregations of individuals regularly distributed one next to the other. The snails have a rich epibiota growing on their shells. This epibiota consist mostly of algae together with acorn (balanomorpha) and goose (lepadomorpha) barnacles, polychaetes, amphipods, bivalves, limpets, and bryozoans that moisten and shade the snails and incidentally give them protection against the sun and the predators. The aggregations of *C. spirata* are therefore protected from high temperatures and desiccation, the two most restrictive physical factors throughout the long periods of exposure during the low tides in the rocky intertidal.

There is strong evidence that wave intensity is an important agent of shell-size variation in intertidal gastropods. Recently, Gómez et al. (2001) reported the variation in shell-thickness of the limpet *Notoacmea biradiata* due to wave intensity, being the shell thicker in exposed rocky shores. The shell size of *C. spirata* was bigger at the most wave-exposed areas compared with the wave-protected areas of the beach. The same condition has been reported for the turbinid snail *Astraea undosa* in 2 localities of Bahía Tortugas, Baja California Sur, México (Gluyas-Millán et al. 1999) and for the trochid snail *Monodonta labio* in the Pacific Coast of Central Japan (Iijima 2001). *Calyptrea spirata*, is a tenacious snail very well adapted to high wave energy habitats, particularly in the rocky intertidal and shallow subtidal zones of exposed coasts. Specimens are found in the most surf-beaten rocks (Keen 1971) where strong waves and currents are definite factors. The wide shell aperture and extensive foot gives to the organisms a better

attachment to the rocky substratum. The irregular margin of the shell adapts well to the irregular microtopography of the rocky substratum, so the water trapped inside during the low tide periods prevents from desiccation and high temperatures, giving oxygen supply for low metabolism. During the periods of low tide, when *C. spirata* is exposed to desiccation and strong variations in temperature, the snails remain strongly attached to the rocky substratum apparently retaining seawater in the cavity of the mantle. Water retention is an important mechanism to avoid an excessive increase in body temperature and allows minimal gas exchange during these periods of low activity and metabolism. Therefore, many intertidal gastropods species change the size of the shell in response to desiccation and temperature (Lowell 1984, Branch 1985). Vermeij (1973) reported a negative relationship between shell size and intertidal height. However, in this study, the shell size of *C. spirata* was not affected by its intertidal position; it is probable that desiccation and temperature do not sufficiently affect the gastropods so their shells do not express a morphologic response to these factors.

In conclusion, the results of this study suggest that the density of *C. spirata* was significantly different between intertidal zones, increasing from the upper to the lower areas of the beach of Teopa, Jalisco. However, there is no gastropod density difference between the sheltered and the exposed parts of the beach. On the other hand, the shell-size variation was associated with wave intensity, with bigger shells (greater shell diameter) being found in the most exposed areas of the beach compared with the protected areas. Although the bigger shells were found in the lower intertidal zone, shore height did not show a significant effect on the size of the shell for *C. spirata*. Individual growth rate of snails smaller than 3 cm of diameter was higher than the rate of larger individuals. The larger snails inhabiting the upper intertidal had significant differences in growth rate when comparing exposed to protected conditions. In contrast, the smaller snails, showed no difference in the growth rate related to wave exposure. Finally, shore height did not show a significant effect on growth rate, because individuals from the upper and lower intertidal zones have similar growth rates. Of many possible factors, shore height, wave intensity, and human impact might be at least partly responsible for these differences. This study, however, was conducted during one season only. Thus, more research is necessary to verify whether these patterns remain common over several seasons. Furthermore, the significance of other biotic factors such as gastropod density, natural predation, and competition should also be taken into consideration in future studies, together with physical factors, principally the desiccation and temperature gradients through the intertidal zone during the low tide periods.

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SIZE SPECIFIC FECUNDITY OF RED ABALONE (*HALIOTIS RUFESCENS*): EVIDENCE FOR REPRODUCTIVE SENESENCE?

LAURA ROGERS-BENNETT,^{1,2,*} RICHARD F. DONDANVILLE³ AND JERRY KASHIWADA⁴

¹California Department of Fish and Game and ²U.C. Bodega Marine Laboratory, PO Box 247, Bodega Bay, CA 94923; ³5342 Winding View Trail, Santa Rosa, CA. 95404; ⁴CDFG, 19160 S. Harbor Dr., Fort Bragg, CA 95437

ABSTRACT The fecundity of wild red abalone, *Haliotis rufescens*, was examined during four reproductive seasons (2000–2003) in northern California. A broad size range of abalone were sampled ($n = 425$) from Van Damme State Park and the Point Arena area. Sexual maturity was defined as the presence of sperm or mature oocytes 170–190 μm in diameter, with a jelly coat, detached from the trabeculae in the gonad. Histologic examination revealed that abalone <50 mm in shell length had not yet sexually differentiated and that 50% of the females from 105–130 mm and the males 75–95 mm had mature gametes while all larger animals were mature. Fecundity, as measured by an estimate of the number of mature eggs per female (X), increased exponentially with increasing shell length (Y) until the peak at 215 mm in shell length after which mature egg number began to decline. The largest female 260 mm (10.24 inches) had >80% necrotic eggs. The data were fit to a non-linear Gaussian curve with 3 parameters;

$$(Y) = Ae^{-(X-\mu)^2 / 2\sigma^2}$$

the maximum productivity ($A = 2,850,000$ eggs y^{-1}), size at maximum productivity ($\mu = 215$ mm), and standard deviation ($\sigma = 38$ mm) of the distribution of maximum productivity versus size. We conclude that whereas large females in excess of 215 mm in shell length undergo some senescence (decline in egg production), these females could potentially contribute as much to reproduction as the mid-size (130–215 mm) females. This suggests that management strategies that protect large females such as marine protected areas or *de facto* reserves will help maintain egg production and that more work is needed to better understand the relationship between female size and egg necrosis.

KEY WORDS: abalone reproduction, *Haliotis rufescens*, size at maturity, necrotic eggs, molluscs, northern California

INTRODUCTION

A basic understanding of reproduction and other vital rates for red abalone, *Haliotis rufescens* (Swainson 1822), populations is necessary for both ecologic studies and fishery management of wild populations. Management strategies such as minimum legal sizes depend on reproduction occurring prior to the onset of fishing. Knowledge of size specific fecundity and size at maturity can be used in size-structured models to examine the population dynamics of wild stocks. Whether the largest oldest females contribute to reproduction, undergo partial or complete reproductive senescence has important implications for population dynamics and management.

Red abalone populations in northern California support an important recreational fishery. Unlike the southern abalone fishery, which was closed in 1996 following intensive fishing (Dugan & Davis 1993, Karpov et al. 2000), the northern fishery is very active with landings estimates ranging from 400,000 to 700,000 red abalone taken per year (CDFG unpubl data). This northern fishery is unique in several ways (1) the use of SCUBA and commercial fishing has been prohibited for more than 40 years; (2) there are few access points along the rugged coastline; and (3) there are long periods of rough ocean conditions. The fishery in the north with an estimated 40,000 fishers is also managed using a combination of a minimum size limit (178 mm), season closures (December to March and July), daily (3/day) and yearly bag limits (24/year), and closed areas. The fishery generates a yearly average of \$13.2 million (1985–1989) in tourism revenue to northern coastal communities (CDFG 1997). As with many fisheries, the recreational abalone fishery highly prizes large trophy animals.

Previous abalone reproduction studies have identified a number of important features associated with wild red abalone in northern California. First, there may be a difference in the size at maturity between males and females (Giorgi & DeMartini 1977); second, red abalone are capable of being highly fecund producing more than 12 million eggs (Giorgi & DeMartini 1977); and third, necrotic eggs have been found in mature females (Young & DeMartini 1970, Giorgi & DeMartini 1977). Laboratory conditioning (feeding kelp) has been shown to reduce the size at first maturity and increase estimates of fecundity by 260%, relative to wild abalone (Ault 1985). These results suggest that reproduction studies based on laboratory (or aquaculture) reared red abalone may not be relevant for wild populations. It is unknown whether the largest females have more necrotic eggs suggesting the possibility of reproductive senescence, as has been found in some other mollusks (Downing et al. 1993).

Red abalone fecundity and size at maturity was investigated for wild animals in northern California. Red abalone populations are centered along 480 km of rocky coastline dominated by bull kelp, *Nereocystis*, in Sonoma and Mendocino Counties north of San Francisco. Samples of red abalone were collected from Van Damme State Park ($n = 393$) over 4 years (2000–2003) and from the Point Arena Abalone Derby ($n = 32$) in 2000 and 2001. For each abalone, gonad volume was determined and samples of gonad tissue were prepared for histologic assessment of reproductive condition. Histologic evaluations were conducted to determine the maturation stage of the ovaries and testes. The potential fecundity of each female was estimated by multiplying estimated gonad volume by the mean number of mature eggs enumerated per slide excluding the necrotic eggs. We discuss the relationship between abalone size, and fecundity as well as reproductive senescence and it's implications for the ecology and management of red abalone populations in northern California.

*Corresponding author. E-mail: rogersbennett@ucdavis.edu

MATERIALS AND METHODS

Collections

A total of 393 red abalone ranging in shell size from 29–224 mm were collected at Van Damme State Park, CA (lat. 39°16'08"N, long. 123°47'58"W) with 137 in April 2000, 71 in January 2001, 62 in February 2002, 123 in January 2003, and 8 in September 2003 (Fig. 1). Sublegal red abalone ranged in size from 29–224 mm in shell length from Van Damme State Park and 212–260 mm from the Point Arena Abalone Derby. Red abalone were collected using SCUBA from depths ranging between 5 and 15m.

The fecundity of the smallest animals (<150 mm) was examined using abalone collected in January and September 2003. The fecundity of the largest red abalone was examined by sampling at the annual Point Arena Derby where recreational fishers skin dive (no SCUBA) for the largest abalone from Sonoma or Mendocino County. The Derby was held August 5, 2000 ($n = 18$), and August 4, 2001 ($n = 14$). Data were not included from the Derby in August 2002, because only three abalone were entered. The Derby was not held in 2003.

Laboratory Dissection and Preparation

All animals were weighed and the length of the shell was measured then, the foot and organs were detached from the shell (shucked) and weighed. Sex was determined by visual examination of the gonad and later confirmed histologically. Mature female

gonad tissue appears dark green in color whereas the male gonad is tan. The length and width of the conical gonadal appendage, including the inner digestive gland core was measured. Slices were made half way down the appendage and the height and width of the gonad/digestive cone and the height and width of the inner core of the digestive gland were measured (Fig. 2). The digestive gland is dark brown in color. Gonad dimensions for animals <125 mm shell length were measured microscopically with an ocular micrometer. Gonad volume was estimated by assuming that the digestive gland and gonad were cone shaped. The volume of the inner digestive gland cone was subtracted from the total cone volume to yield the volume of the outer gonad cone as described by Tutschulte (1976), and Tutschulte and Connell (1985). The gonad index was defined as gonad volume *100/abalone body weight.

Samples of the gonad were taken for histologic preparation from the mid-section. All gonad samples were fixed in invertebrate Davidson's solution (a formalin based fixative) (Shaw & Battle 1957) for 24 hours, then transferred to 70% ethanol. Once in alcohol the tissues were processed for paraffin histology. Deparaffinized 5- μ m sections were stained with hematoxylin and eosin (Luna 1968), and mounted on slides for examination using light microscopy. Not all samples had enough gonad tissue to score and some abalone tissue sections were not quantifiable and so we report the histologic results from 86 abalone in April 2000, 62 from January 2001, 62 from February 2002 and 123 from 2003.



Figure 1. Map of northern California showing Van Damme State Park and Point Arena where red abalone were collected for this study.

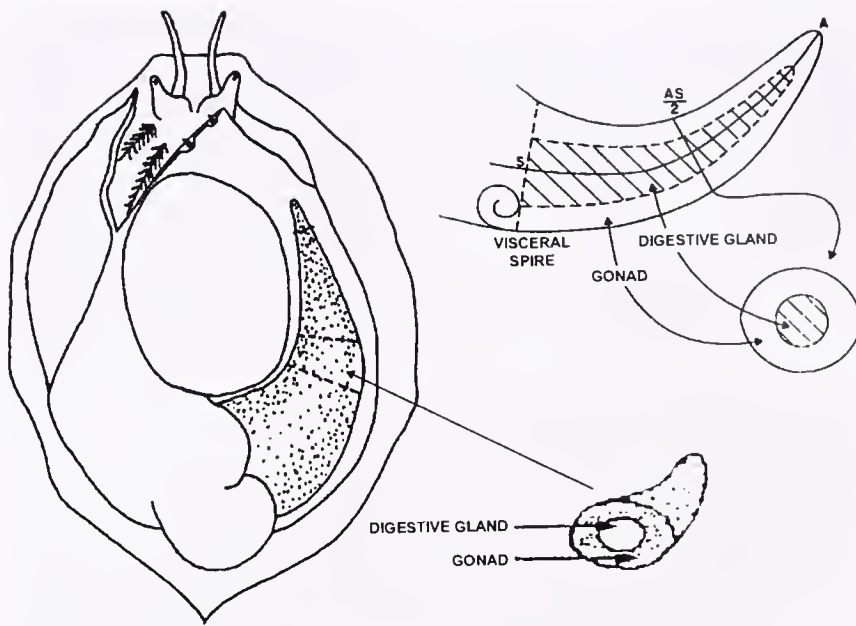


Figure 2. Diagram of red abalone anatomy showing dissection and measurements of red abalone gonad and digestive gland within conical appendage.

Classification of Oocytes

Three classes of oocytes were identified: immature, mature, and necrotic (degenerating) (Young & DeMartini 1970, Brickey 1979, Martin et al. 1983). Immature oocytes range from 5–60 μm in diameter, stain violet, and attach to trabeculae in the gonad. The larger immature oocytes, 40–60 μm diameter become pear shaped and have a distinct nucleus. Slightly larger oocytes, approaching mature size but still attached to trabeculae, were classified as immature. Mature oocytes were 170–190 μm diameter and approximately circular.

They have a speckled, densely granular appearance due to a combination of colorless lipid droplets, <6 μm diameter, set in a magenta-stained yolk groundmass. Lipid droplets comprise 30% to 50% of the oocyte volume. The nucleus is usually crescent shaped. The oocytes are surrounded by three extra-cellular layers: a 1- μm vitelline layer, a chorion about 10 μm thick, and the jelly coat 10–20 μm thick. Mature oocytes are detached from the trabeculae, free to be released at spawning.

Young and DeMartini (1970) and Giorgi and DeMartini (1977) first described necrotic oocytes that appear to be autolysing residual mature oocytes. The necrotic oocytes are approximately the same diameter as mature oocytes and are composed of irregular, textureless, orange eosinophilic masses up to 50- μm wide and set in a fine mesh of dark purple basophilic material. Numerous vacuoles up to 30- μm diameter connect to give the oocytes a spongy, mosaic appearance. In the early stage of necrosis the oocytes are the same size and shape as mature oocytes. In more advanced stages, the oocyte wall distorts and ultimately ruptures, dispersing the cell contents into the gonad.

Fecundity Estimates

In addition to the gonad volume the total number of mature eggs was determined for each abalone. Egg number was estimated by counting all the mature eggs in 4 microscope fields ($\times 200$), dividing by the volume of the 4 fields, and multiplying by the

gonad volume. The volume of a microscope field is equal to the area of the field times the thickness. The area was calculated using an ocular micrometer for field dimensions. Because a small mature oocyte section can be from an oocyte largely above or below the section plane, the thickness was defined as twice the average oocyte diameter. The average oocyte diameter was 176 μm , exclusive of the jelly layer. It was determined by measuring the diameter of 1000 of the largest, roundest oocytes in the sections, and by measuring fresh oocytes.

These methods were used to estimate the number of immature eggs in the smaller females, <125 mm and necrotic eggs in all size females. For abalone <76.6 mm all oocytes were counted. Due to the dramatic increase of the number of oocytes in abalone >76.6 mm, only larger oocytes, 40–60 μm were counted, because smaller oocytes were too numerous to count. Necrotic egg number was estimated by counting all the necrotic eggs in 4 microscope fields ($\times 200$), dividing by the volume of the 4 fields, and then multiplying by the gonad volume.

RESULTS

Size at Maturity

Abalone shell length was related to reproductive maturity (Fig. 3 and 4). The smallest female with mature oocytes capable of spawning was 106 mm long and weighed 170 g. The smallest animal that could be identified as a female with immature oocytes had a shell length of 50 mm, and a body weight of 15 g. Animals less than 50 mm did not have differentiated gonad tissue. There were 19 immature females ranging from 50–104 mm. Only 6 of 17 females between 106–126 mm had mature oocytes. All 48 females from 129–175 mm had mature oocytes.

Mature males were found at a smaller size than females. The smallest male with sperm was 75 mm and weighed 40 g. The smallest male with spermatocytes and testis structure was 64 mm, and weighed 30 g. Of eight males in the 75–91 mm size range four of them had sperm. All the males between 91–175 mm had sperm ($n = 63$) with three exceptions that were large males (collected April 2000) with well developed testis structure and spermatocytes.

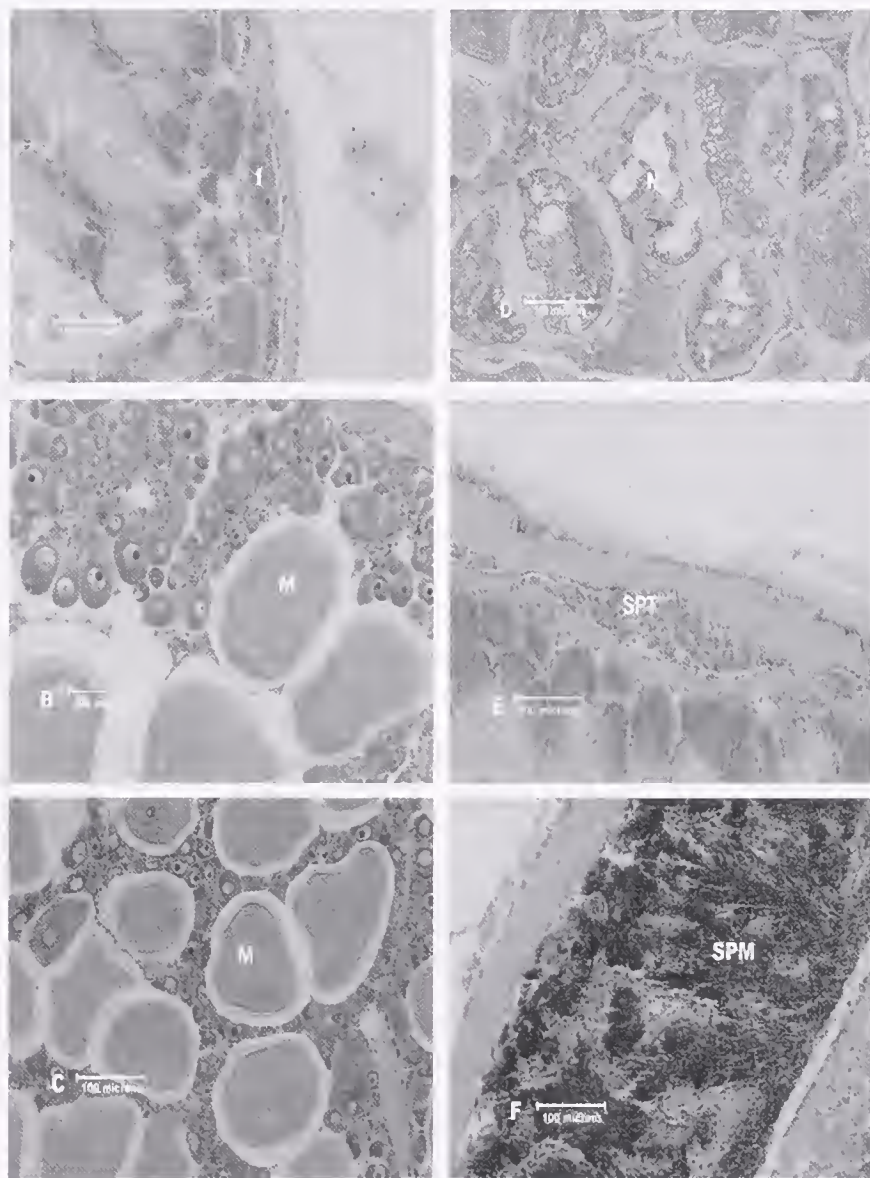


Figure 3. Photomicrographs of gonadal stages of red abalone, *H. rufescens*. A. Female with immature (I) oocytes, shell length 50 mm. B. Female with mature (M) oocytes shell length 106 mm. C. Female with abundant mature oocytes shell length 130 mm. D. Female with necrotic (N) oocytes shell length 187 mm. E. Immature male with spermatocytes (SPT), no sperm shell length 75 mm. F. Mature male with abundant sperm (SPM) shell length 100 mm.

cytes, but no observable sperm, suggesting they spawned prior to collection.

Size Specific Reproduction

Gonad volume increased with increasing shell size after 50 mm shell length for females and 64 mm for males. No gonad tissue was visible for abalone smaller than 50 mm in shell length using the microscope. Gonad tissue, or tissue destined to become gonad, appeared as a thin purplish membrane around the digestive gland for animals 50–75 mm. Gonad indexes and sex for all abalone <112 mm (and some abalone in the 114–126 mm size range) with these thin membrane-like gonads were determined from the histologic sections. Abalone >129 mm were measured macroscopically during dissection. These measurements revealed that females ranging in size from 50–75 mm had very thin sections of gonad (<50

μm) with few immature eggs averaging 801 immature eggs per female ($n = 3$). For example, within the three gonad cross sections of a 65-mm female; two sections had no oocytes; whereas the third had only three immature oocytes. Females greater than 75 mm in shell length had dramatically thicker gonad tissue layers (>250 μm) accompanied by a large increase in the numbers of immature eggs averaging 725,763 immature eggs for females 75–100 mm ($n = 8$).

Egg number increased rapidly until a shell length of 215 mm when mature egg number declined (Fig. 4). The relationship between shell length (X) and egg number (Y) can be modeled using a Gaussian curve of the form:

$$(Y) = Ae^{-(X-\mu)^2/2\sigma^2}$$

where A is defined as the maximum productivity, μ is the size at maximum productivity, and σ the standard deviation describes the

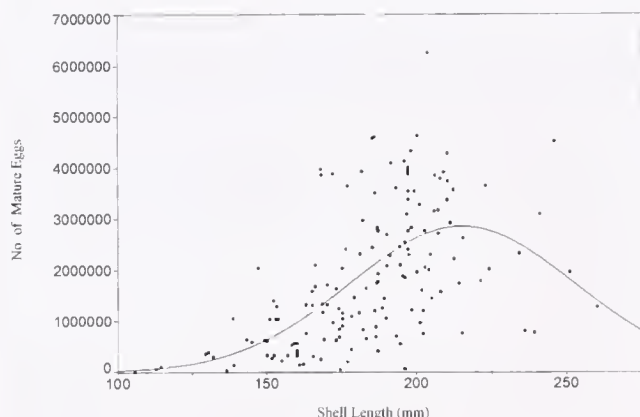


Figure 4. Graph of the relationship between mature egg number and adult female red abalone size ($n = 154$) from Van Damme and Point Arena in northern California modeled using a Gaussian curve. One female (233 mm length) was outside the 99.9% prediction interval with more than 11 million eggs and was excluded from the Gaussian curve fit.

width of the distribution of maximum productivity versus size. Females are subject to fishing pressures from 177 mm to 253 mm (± 1 SD) and this size class is potentially responsible for 68% of the total mature egg production whereas sublegal females will produce 32% of the egg production.

The smallest mature female (105 mm) had the fewest mature eggs ($\approx 2,400$) whereas the largest females had the most eggs (Table 1). Mature egg number increased from an average of 63,000 for females ranging in size from 90–120 mm to 2.5 million eggs per female from 178–220 mm and 3 million for the largest females >220 mm. The average number of mature eggs increased per gram of body weight from 330 for females from 90–120 mm in shell length to almost 2,500 eggs per gram of body weight for females 178–220 but declined to 2,000 eggs per gram of body weight for the largest size class >220 mm. In contrast to the small females with few oocytes, the small males (75 mm), had abundant sperm (Table 2).

Necrotic Eggs

The largest red abalone had high percentages of necrotic eggs (Table 3, Figs. 3 and 4). The largest red abalone in the collection (260 mm) from the sport derby had $>80\%$ necrotic eggs. The smaller abalone (80–120 mm) had the least number of necrotic eggs.

DISCUSSION

Abalone Size and Reproduction

Histologic examination of gonad tissues revealed that reproductive stage was related to shell length (see Table 3, Fig. 3). Red abalone became sexually differentiated at approximately 50 mm when primary oocytes or spermatocytes (or sperm) are first observed in the gonad tissue. In females >75 mm in shell length, there was a dramatic increase in the thickness of the ovary tissue accompanied by an exponential increase in the number of primary oocytes (see Table 3). The smallest female with mature oocytes was 105 mm, although it was not until a shell size of 130 mm that 100% of the females had mature eggs and the number of mature eggs increased dramatically. Egg number increased to a peak at an abalone shell length of 215 mm, followed by a decrease in mature

TABLE 1.

Sexual maturity of female red abalone ranging from 28.8–150.0 mm in shell length collected from Van Damme State Park in January and September 2003. Total number of mature oocytes estimated for each female abalone. Plus signs (++) indicate the presence of immature or mature eggs while minus signs (--) indicate their absence. Not Measured, not enough tissue to measure (NM). Indeterminate Sex (IS).

Shell Len. (mm)	Body Wt. (gm)	Total No. Mature Oocytes	Immature Oocytes Present	Mature Oocytes Present
28.8	1.6	0	IS	--
36.4	4.1	0	IS	--
36.8	4.2	0	IS	--
46.3	12.0	0	IS	--
49.8	15.0	0	++	--
51.5	15.0	0	++	--
56.5	20.0	0	IS	--
57.7	21.0	0	++	--
58.0	18.0	0	++	--
58.5	14.0	0	IS	--
59.8	24.0	0	++	--
61.2	24.0	0	IS	--
61.5	25.0	0	++	--
62.5	30.0	0	IS	--
64.6	31.0	0	++	--
76.6	56.0	0	++	--
76.7	49.0	0	++	--
81.8	53.0	0	++	--
84.0	68.0	0	++	--
85.0	66.0	0	++	--
85.2	64.0	0	++	--
90.6	90.0	0	++	--
91.0	87	0	++	--
96.2	112	0	++	--
98.4	136	0	++	--
101.8	117	0	++	--
103.9	134	0	++	--
105.7	170	2400	++	++
107.1	120	0	++	--
109.2	180	0	++	--
111.4	187	0	++	--
112.8	152	74000	++	++
114.5	234	112000	++	++
116.4	182	0	++	--
118.5	163	0	++	--
118.7	232	NM	++	++
119.4	261	NM	++	++
120.1	127	0	++	--
121.1	254	0	++	--
122.0	311	0	++	--
122.0	156	0	++	--
125.0	207	0	++	--
125.0	272	NM	++	++
126.4	257	0	++	--
129.2	306	372000	++	++
130.4	262	398000	++	++
132.0	361	308000	++	++
136.3	292	33000	++	++
138.5	391	1073000	++	++
138.8	383	144000	++	++
141.0	237	NM	++	++
142.9	341	NM	++	++
143.0	528	645000	++	++
145.0	482	597000	++	++
147.0	499	2035000	++	++
149.0	499	627000	++	++
150.0	585	334000	++	++
150.0	408	606000	++	++

TABLE 2.

Sexual maturity of male red abalone ranging from 28.8–140.1 in shell length collected from Van Damme State Park in January and September 2003. Plus signs (++) indicate the presence of spermatocytes or mature sperm while minus signs (--) indicate their absence. Indeterminate Sex (IS).

Shell Len. (mm)	Body Wt. (gm)	Spermatocytes Present	Mature Sperm Present
28.8	1.6	IS	
36.4	4.1	IS	
36.8	4.2	IS	
46.3	12	IS	
56.0	17	++	--
56.5	20	IS	
58.5	14	IS	
61.2	24	IS	
62.5	30	IS	
64.4	30	++	--
72.2	40	++	--
72.3	39	++	--
74.9	40	++	++
75.8	46	++	++
78.0	71	++	--
81.6	72	++	++
82.8	60	++	--
86.3	66	++	++
90.8	78	++	--
91.0	90	++	--
94.7	104	++	++
94.9	88	++	++
99.5	102	++	++
100.8	108	++	++
102.1	143	++	++
103.8	138	++	++
104.9	130	++	++
105.6	162	++	++
105.9	127	++	++
106.9	143	++	++
111.3	154	++	++
111.3	151	++	++
113.7	213	++	++
114.7	160	++	++
115.8	207	++	++
117.6	226	++	++
120.4	280	++	++
120.4	220	++	++
123.8	242	++	++
123.9	202	++	++
127.0	347	++	++
127.8	276	++	++
129.0	291	++	++
129.3	320	++	++
129.5	275	++	++
131.7	304	++	++
132.3	323	++	++
135.0	307	++	++
135.1	282	++	++
138.0	308	++	++
138.8	300	++	++
140.1	288	++	++

egg number as abalone size increased due to an increase in the number of necrotic eggs (Table 3 and Fig. 4). The peak number of eggs estimated from the Gaussian curve was 2,850,000 eggs, although many individuals had more eggs than the peak of the curve and one individual had in excess of 11 million eggs. Females from this same site, however, have been estimated to produce up to 23 million eggs (Rogers-Bennett et al. MS in prep). The largest females had the highest percentages (80–90%) of necrotic eggs (see Table 3 and Fig. 4).

Size at First Maturity

Other studies examining the size at first reproduction support our results. Giorgi and DeMartini (1977) also found that small female abalone 40–50 mm in length had immature oocytes (see Table 3, Fig. 3), however different criteria were used to define maturity. We define maturity as requiring the presence of mature oocytes. Mature oocytes can be distinguished from immature (<50 μ m in diameter) based on egg size (180 μ m in diameter), having both stained yolk and lipid droplets and being detached from the trabeculae (stalk), ready to be spawned (see Fig. 3). Samples collected in this study in January and September 2003 showed that the smaller abalone 50–100 mm did not have mature oocytes. Giorgi and DeMartini (1977) also found that abalone <132 mm collected in June and July only had small (<50 μ m) immature eggs. Ault (1985) collected wild red abalone and found that only female red abalone >110 and males >60 mm spawned successfully after artificial induction (using hydrogen peroxide) further supporting our conclusions.

There was a large gap in sizes between immature and mature males and females (Tables 1 and 2). Females with immature oocytes were 50 mm in shell length and those with mature oocytes were 130 mm. This suggests two alternative hypotheses: either the eggs from smaller females mature at a different time of year, which we missed with our sampling dates (January and September 2003) or it may take a few years before ovaries mature. Eggs from female frogs, *Rana temporaria*, take 3 years to mature (Grant 1953). A Gaussian growth curve has been used to estimate that it would take 3–4 years for females in northern California to grow from 50 mm to maturity at 105 mm and 1–2 years for males to mature from 50 to 75 mm (Rogers-Bennett et al. MS in review).

We found a significant difference in the size at maturity between the sexes with females maturing at a larger size than males, as was found in the southern California (Tutschulte 1976, Tutschulte & Connell 1985). Wild abalones in southern California have also been found to mature at large sizes. Tutschulte (1976) found that 4 of 19 pink abalone in the size ranges from 59–119 mm had mature oocytes, and that green abalone mature at 101 mm. More studies however, are needed on the time and food resources required for maturation of gametes in wild populations.

Oceanographic conditions are known to influence abalone reproduction and successful recruitment (Shepherd et al. 1998). For example, El Niños are associated with decreased dissolved nitrogen levels, poor kelp growth (Tegner et al. 1997), poor abalone growth (Haaker et al. 1998) and possibly decreased abalone egg production. Perhaps as a result of oceanographic conditions recruitment is highly variable in time and space (McShane & Smith 1991). If females have just a few years to spawn prior to growing into the fishery, some females may not spawn during this time if oceanographic conditions remain unfavorable. In fact, minimum size limits failed to prevent the collapse of abalone populations in

TABLE 3.

Fecundity estimates for female red abalone from Van Damme and Point Arena by size class showing average estimated gonad volume (EGV), average number of immature, mature, necrotic eggs. Percent of necrotic eggs to total number of mature and necrotic eggs per female abalone.

Size Class (mm)	No. Animals in Class	Ave. EGV mm ³	Ave. No. Immature Oocytes	Ave. No. Mature Oocytes	Ave. No. Necrotic Oocytes	% Necrotic Oocytes
0-40	(N = 10)	—	0	0	0	0
41-80	(N = 13)	80.7	37721	0	0	0
81-120	(N = 20)	1237	2027000	62959	28024	30.8
121-150	(N = 21)	6328	NM	597706	59076	9.0
151-178	(N = 49)	12623	NM	1153328	75154	6.1
179-220	(N = 80)	26362	NM	2491901	192538	7.1
221-260	(N = 11)	44746	NM	3068098	2005563	40.0

southern California (Tegner et al. 1989, Karpov et al. 2000) and elsewhere (Shepherd & Rodda 2001, Shepherd et al. 2001).

Evidence for Limited Reproductive Senescence

The largest individuals had the most (80% to 90%) necrotic eggs (Table 3, Fig. 4) suggesting the onset of reproductive senescence. On average, individuals in the largest size class had 40% necrotic eggs, the most of any of the size classes (see Table 3). Legal size abalone from this same date had less than 20% necrotic eggs. Histologic examination of female ovaries ($n = 34$) from VDSP indicated that in August 2001 only 12% of the eggs were necrotic, suggesting August is not a period of unusually high percentages of necrotic eggs. Few studies however, have examined reproductive senescence in mollusks, with freshwater mussels showing reproductive senescence (Downing et al. 1993), whereas marine quahog clams did not (Walker 1994).

Egg production capacity of very large female abalone (>215 mm) is still high, despite having high percentages of necrotic eggs. The size class with the greatest number of mature eggs per female abalone was the largest size class (220–260 mm) (see Table 3, Fig. 4), suggesting that even with reproductive senescence, the largest females are still capable of producing millions of eggs. It is unknown, however, if these females spawn successfully relative to smaller females or if the eggs produce viable, high quality larvae.

Egg Conservation and Reserves

One way to maintain egg production would be to exclude large females from the fishery using upper size limits or area closures. Legal size abalone (± 1 SD of the peak 215 mm) have the potential to generate 68% of the egg production (see Fig. 4). As in other recreational trophy fisheries however, where large animals are highly prized, the addition of an upper size limit may not have popular support. While reserves may benefit the fishery in the

north, these areas were largely absent in the south where stocks collapsed. The south also had additional abalone fishing pressures compared with the north because much of the coast is accessible, ocean conditions are frequently favorable and the use SCUBA was permitted for abalone fishing. The few reserves in the south such as the Anacapa Island Ecological Reserve (McArdle 1997) did function to protect large abalone and was shown to have the potential for increased egg production compared with neighboring fished areas (Rogers-Bennett et al. 2002).

The fishery in the north seems to be sustainable, perhaps in part due to protection of a portion of the stock in reserve areas and a large *de facto* reserve (both at deep depths [<8.5 m] and along inaccessible portions of the coast), which may be the key for sustainability as has been suggested in other fisheries (Walters & McGuire 1996). Inside established reserves in the north more juvenile abalone have been found compared with fished areas (Rogers-Bennett & Pearse 2001). If large abalone are to be protected and allowed to spawn, then maintaining reserves will need to remain a top priority for management, recreational divers and wildlife protection.

ACKNOWLEDGMENTS

Thank you to the recreational abalone divers along the north coast who participated in the study and allowed us to sample their abalone. Thanks to all the abalone creel samplers and the dive team of the California Department of Fish and Game. The authors thank Carolyn Friedman, Jim Moore, Thea Robbins, and Beverly Braid for their histologic assistance. Thanks to Patty Wolf and Eric Larson of the CDFG. This work was funded in part by the Recreational Abalone Advisory Committee and the recreational abalone stamp. The Bodega Marine Laboratory provided logistic support. Contribution number 2199, Bodega Marine Laboratory, University of California Davis.

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THE SETTLEMENT OF ABALONE (*HALIOTIS DISCUS HANNAI* INO) LARVAE ON CULTURE LAYERS OF DIFFERENT DIATOMS

NURIT GORDON,¹ MUKI SHPIGEL,¹ SHEENAN HARPAZ,² JOHN J. LEE³ AND AMIR NEORI^{1*}

¹Israel Oceanographic and Limnological Research Ltd., National Center for Mariculture, P.O. Box 1212, Eilat 88112, Israel; ²Department of Aquaculture, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel; and ³Department of Biology, City College of City University of New York, Convent Ave at 138 St, New York, New York 10031

ABSTRACT An innovative method was developed to quantitatively measure the attractiveness of cultured diatoms for early stages of abalone larvae settlement. Larvae of the abalone *Haliotis discus hannai* were offered, in a petri dish, a choice of several food patches, each made of a diatom monoculture layer. The distribution of the larval settlement on the patches of 17 diatom species was determined. *Nitzschia laevis* induced the most abalone larvae settlement, followed by *Navicula cf. lenzii* and *Amphora luciae*. The attractiveness of these diatoms for abalone larvae settlement was not directly related to algal biochemical composition or algal color. It is suggested that compounds excreted by algal cells determine their inductive properties for larval settlement. The larval chemosensory response depends on the concentration of these inductive substances. Sonicated diatom cell suspensions strongly induced larvae attachment, whereas dilute suspensions did not. Boiling settlement-inductive sonicated diatom cell suspensions did not affect their induction ability, implying that native proteins are not essential for the response. Aspartic acid was the only free amino acid excreted by two tested diatoms. It was also the only pure amino acid found inductive for larval settlement. It is therefore suggested that aspartic acid is a component of the settlement induction complex.

KEY WORDS: abalone, *Haliotis discus hannai*, larvae settlement, larval feeding, abalone, diatoms, mariculture

INTRODUCTION

The process of abalone larval settlement consists of several characteristic behaviors. Descent and exploration of the settlement substrata by the swimming larva is followed by attachment, which includes inspection and orientation at a favorable spot, and is concluded finally by metamorphosis (Seki & Taniguchi 1996, Seki 1997). The induction of larval settlement is a most critical stage in abalone recruitment in the wild and in seed production in culture. Abalone larvae require highly specific cues for attachment and metamorphosis stimulation (Morse & Morse 1984, Morse 1985, Roberts 2001). The absence of cues lead to low settlement rates and low survival in early post larval growth stages (Searcy-Bernal et al. 1992, Slattery 1992, Roberts et al. 1999, Daume et al. 1999, Takami et al. 2002). A better understanding of the settlement processes and the identification of a reliable approach for efficient recruitment and early growth in abalone larvae, are therefore of paramount importance for furthering both abalone recruitment research and abalone commercial reproduction.

Substances that have been reported to induce settlement in abalone larvae are crustose and coralline algae (Morse & Morse 1984, McShane 1992, Shepherd & Turner 1985), diatom layers (Slattery 1992, Takami et al. 1997, Daume et al. 1999), chemicals like GABA [gamma-amino butyric acid] (Morse 1985, Trapido-Rosenthal & Morse 1986, Morse & Morse 1984, Morse & Morse 1988), abalone mucus trails (Slattery 1992, Searcy Bernal et al. 1992, Seki & Taniguchi 1996, Seki 1997, Takami et al. 1997,

Bryan & Qian 1998), and bacterial films (Bryan & Qian 1998, Roberts 2001).

The induction of the settlement process in larval abalone *Haliotis rufescens* on crustose red macroalgae (such as *Lithothamnium californicum*) is based on chemosensory recognition of morphogenic and regulatory molecules (such as amino acids), occurring on the macroalgae outer surfaces and the surrounding water (Morse & Morse 1984, Trapido-Rosenthal & Morse 1986, Morse 1992).

Naturally recruited diatom films have long been used to induce larval settlement in abalone hatcheries around the world, but with a limited awareness or management of suitable settlement-inductive species and the properties that make them attractive to abalone larvae (Roberts 2001). Understanding the factors that regulate effective settlement, post larval growth, and the selection of cultivable diatoms that promote it, are therefore essential (Matthews & Cook 1995, Roberts 2001).

Diatoms release into their surrounding water substances that can induce settlement (Helebust 1974, Admiraal et al. 1986, Searcy-Bernal et al. 1992). It is hypothesized that the selective settlement of abalone larvae on a film of a certain species of algae is associated with larval-attracting chemicals, which "attractive" algae have on their cellular exterior or secrete into the water around them (Kuehn 1997). Such chemicals can create gradients detectable by small aquatic animals even in turbulent water (Finelli et al. 1999), and abalone larvae are known to have the physiologic ability to sense them (Morse & Morse 1984, Morse & Morse 1993, Morse 1992, Baxter & Morse 1992). This article describes a method developed to quantitatively measure the preference of swimming larvae for attachment onto a range of cultured diatoms (the term "settlement" is used henceforth, even though metamorphosis was not studied). "Attractive" and "not attractive" diatoms were selected by this method and then used to probe the chemical basis for the chemosensory settlement response of abalone larvae.

This work was supported by the Israeli Ministry for National Infrastructures (N.G., M.S. and A.N.), by grants from the European Commission (M.S. and A.N.) and NIH/NIGMS 08168-22 (J.J.L.).

*Corresponding author. E-mail: aneori@shani.net

MATERIALS AND METHODS

Larval Culture

Larvae of *Haliotis discus hannai* were obtained from an in-house abalone hatchery in Eilat, Gulf of Aqaba, Red Sea. Adults were induced to spawn using ultraviolet light (Kikuchi & Uki 1974). Fertilized eggs were collected and transferred into 20-L culture aquaria at a concentration of 12 eggs per mL. Rafamycin antibiotic was added at a concentration of 1.5 mg/L. Hatched larvae were kept at 22°C, with a 12 L: 12 D light cycle (60–70 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$), for 4–5 days, until they were competent for settlement. Larval competency was assessed based on swimming behavior, as described in Seki and Taniguchi 1996. The competent larvae were then used for the settlement preference experiments.

Diatom Cultures

Cultures of benthic diatoms were isolated from local waters and elsewhere (Table 1). Diatom cultures were made axenic by an antibiotic mixture of Penicillin G (800 $\mu\text{g/mL}$), Ampicillin and Streptomycin (400 $\mu\text{g/mL}$) and Chloramphenicol (160 $\mu\text{g/mL}$) for 24 hours (Ucko 1996). The cultures were then transferred into Gentamycin sulfate (200 $\mu\text{g/mL}$) for 24 hours. The axenity of the treated cultures was confirmed by plating them on a rich nutrient agar (DIFCO) petri dishes, which can clearly show any bacterial contamination. The axenic algal cultures were cultured at 22°C to 24°C in a slightly modified f/2 medium (Guillard & Ryther 1962). The cultures were maintained in test tubes under continuous light ($\sim 110 \mu\text{mol photons m}^{-2}\text{s}^{-1}$).

TABLE 1.

The diatoms evaluated in this study. Numbers (in parenthesis) after species names indicate isolates numbers; see Taxonomic Appendix for taxonomic details.

	Algal Species	Source
1	<i>Nitzschia laevis</i> Hustedt 1939	Sediment pond, IOLR, Eilat, Israel
2	<i>Navicula</i> cf <i>lenzii</i> Hustedt 1939	—//—
3	<i>Navicula</i> cf <i>viminoides</i> Giffen 1975	—//—
4	<i>Navicula salinicola</i> Hustedt 1939	—//—
5	<i>Amphora</i> cf <i>tenerrima</i> (1) Aleem and Hustedt 1951	—//—
6	<i>Amphora</i> cf <i>tenerrima</i> (2) Aleem and Hustedt 1951	—//—
7	<i>Amphora</i> sp. (2)	—//—
8	<i>Amphora luciae</i> Chohnoky emend Archibald 1983	Martha's Vineyard Island, lake Tashmoo, Massachusetts
9	<i>Nitzschia laevis</i> Hustedt 1939 (Symbiont clone)	Gulf of Aqaba, Eilat, Israel
10	<i>Amphora</i> sp. (1)	—//—
11	<i>Amphora</i> sp. (4)	—//—
12	<i>Amphora</i> sp. (6)	—//—
13	<i>Amphora coffeiformis</i> Agardh	Ecuador
14	<i>Navicula</i> sp. (1)	Lizard Island, Great Barrier Reef, Australia
15	<i>Navicula</i> sp. (2)	—//—
16	<i>Navicula</i> sp. (3)	Carlsbad farm C/A, California
17	<i>Amphora</i> sp. (3)	—//—

For the settlement preference experiments, algae were axenically grown on a Bacto-agar (DIFCO) solid medium, which was prepared with modified f/2 medium as mentioned earlier, or with artificial sea water (ASW) (Jones et al. 1963) enriched with silica. Petri agar dishes were inoculated by spreading the diatoms uniformly on the agar surface and left to grow for about 2 weeks, when a uniform diatom layer was formed.

The Experimental System, Developed to Quantify Larval Settlement Preference

Preparation of the Experimental Petri Dishes

The larval settlement preference experimental system was a modification of the "cafeteria" method used by Lee et al. (1977). The top of an alcohol-sterilized 16 mm test tube was used to remove diatom-covered agar plugs from the diatom culture dishes and to insert them into the same size holes in Agarose agar (Sigma) layers in the experiment dishes. These were prepared by pouring 25 mL of 1% Agarose agar into 90 mm petri dishes, in thin layers. Preliminary experiments showed that other types of agar negatively affected the settlement and growth of the larvae. After cooling and hardening of the agar, four plugs were cut out and removed from the Agarose agar layer in each dish with an alcohol-sterilized 16 mm test tube. Three of the round empty spaces that remained were filled, as described earlier, with diatom covered Bacto-agar plugs of the same size, whereas the fourth empty space was filled with a control Bacto agar plug, without any algal layer (Fig. 1). Usually 10 replicates were prepared of each experimental dish, with the same three diatom species and a control plug. Special care was taken to reduce microbial contamination during the experiments once the axenity was compromised with the addition of the larvae. The experiments took place in a clean culture room and manipulations took place in a sterile laminar-flow hood.

Assessment of the Settlement Preference of Larval Japanese Abalone onto Layers of Different Diatoms

Forty mL of 32 ppt filtered sea water was carefully pipetted onto the surface of the Agarose agar layer in the experimental dishes, to make aqueous layers (0.6 cm in depth) for the swimming larvae. Competent larvae (200–250) were gently introduced to the center of each dish. The larval density was selected after a set of preliminary tests that suggested this density as optimal for counting and statistics, with no harm to the settlement process. The dishes were left covered, at 22 °C and under continuous light ($\sim 60 \mu\text{mol photons m}^{-2}\text{s}^{-1}$). These conditions involved minimal technical difficulties in the laboratory, and fell within the range of

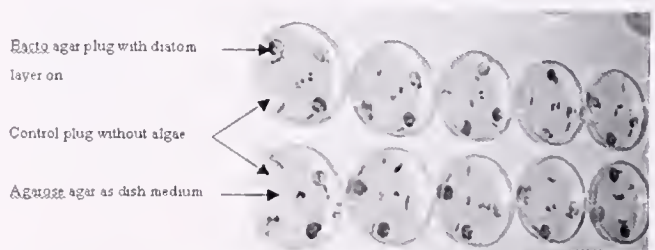


Figure 1. A photograph describing an experiment with 10 replicate petri dishes, used for the measurement of settlement preference by abalone larvae. Each dish contains 3 patches of the same 3 diatoms and a no-diatom control patch (see Materials and Methods for details).

conditions reported in the literature. No preliminary tests were made to optimize temperature and light conditions.

Counts of settled larvae on top of the algal patches were carried out using a dissecting microscope several times during 24 hours. Attractiveness of a certain algal species to the larvae was determined by the number of larvae that settled (even if metamorphosis was not completed) on its patch relative to the number of larvae settled on the other diatom patches and on the control patch up to a certain time (usually 10 h) after initiation of the experiment. Seventeen species of algae were tested for larval settlement preference in 50 experiments, each consisting of 10 replicate petri dishes, each with the same group of three diatoms.

Elucidation of Possible Factors Influencing Settlement Preference by the Larvae of the Japanese Abalone

Settlement Preference of the Larvae onto Sonicated and Boiled Diatom Cells

This experiment used the settlement preference measurement system described above to evaluate a possible linkage between algal internal biochemical composition and larval preference to settle on it. Concentrated algal cells were broken with an ultrasonic cell disruptor (Microson) for 5 minutes (microscopy check assured cell disruption) and then mixed with cooled (40–45 °C), still liquefied Bacto agar. Larval settlement preference was tested towards *Nitzschia laevis* (2.05×10^6 cells/plug), which in its native state had been found very attractive to the larvae, and *Amphora cf. tenerrima* (2) (3.9×10^5 cells/plug), which in its native state had been found to be unattractive or even repulsive to the larvae.

A second experiment was conducted as earlier stated, but the sonicated cells were boiled for 15 minutes before mixing with agar, to denature the proteins. The aim of this experiment was to determine the role of native proteins in attractiveness to the larvae.

Settlement Preference of the Larvae onto Agar Plugs Soaked with Algal Growing Medium and Amino Acids

Settlement preference of abalone larvae was tested towards liquid culture media in which algae had been axenically grown. Algal cultures were centrifuged (3000 rpm for 5 min in a clinical centrifuge) and the clear media were then filtered through a Nucleopore filter (0.2 μ) to remove any cell debris, and used with 1% agar to prepare an agar layer from which plugs could be cut for the assays.

Settlement preference of the larvae was also tested towards agar plugs soaked with solutions (final concentration in agar 10 mM) of the following L-amino acids: methionine, threonine, serine, phenylalanine, tryptophan, glycine, DL-alanine, proline, betaine, leucine, taurine, valine, cysteine, histidine, ornithine, arginine, lysine and aspartic acid (All Sigma reagent grade).

Statistical Analyses

The data were compared by ANOVA (one way) with Duncan's multiple range test. Results in percentages were arcsine square root-transformed prior to ANOVA to homogenise variances (Sokal & Rohlf 1969).

The Effect of Light on the Settlement of Abalone Larvae

Settlement preference of abalone larvae was tested towards agar plugs with three different sonicated algal species (made as

earlier stated). Two series of 10 replicate algal dishes were made. One set was put in the dark, and the other in light.

RESULTS

Assessment of the Settlement Preference of Larval Japanese Abalone (*Haliotis discus hannai*) onto Layers of Different Diatoms

In most cases larvae settled during 10 hours from the experiment initiation (Fig. 2). Strong settlement stimulants, like sonicated cell suspensions, speeded up the settlement to 1.5 hours to 5 hours.

The larval abalone preferred, by a large margin, to settle on patches of *Nitzschia laevis* in each of the experiments in which this diatom was offered (Table 2). On average, almost half the larvae settled on *N. laevis* in each relevant individual experiment, in comparison to much smaller fractions on the other diatoms offered and on the control (Table 3). Significant (1-w ANOVA, $P < 0.05$) preference of the abalone larvae for the diatoms *Navicula cf. lenzii* and *Amphora luciae* was obtained in 82% and 75% of relevant experiments, respectively (see Table 2). On the average, about a quarter to a third of the larvae preferred these two diatoms in each relevant individual experiment (Table 3).

Nitzschia laevis (the symbiont clone) and *Amphora coffeiformis* were also found attractive to the larvae in individual experiments (see Table 3). However, over half of the diatoms tested were not found to be attractive to larval settlement in most of the experiments (Tables 2 and 3).

Settlement of Japanese Abalone Larvae on Sonicated Diatom Cell Suspensions

The larvae preferred by a large margin ($P < 0.001$) to settle onto *Nitzschia laevis* layers rather than onto *Amphora cf. tenerrima* (2) layers or the control (no algae) (Fig. 3). Whole cells of *A. cf. tenerrima* (2) were found unattractive to larvae in this experiment and others (see Table 2). On the contrary, the abalone larvae showed a strong preference (70% of the settled larvae; $P < 0.001$) for settlement onto sonicated cells of the same *A. cf. tenerrima* (2).

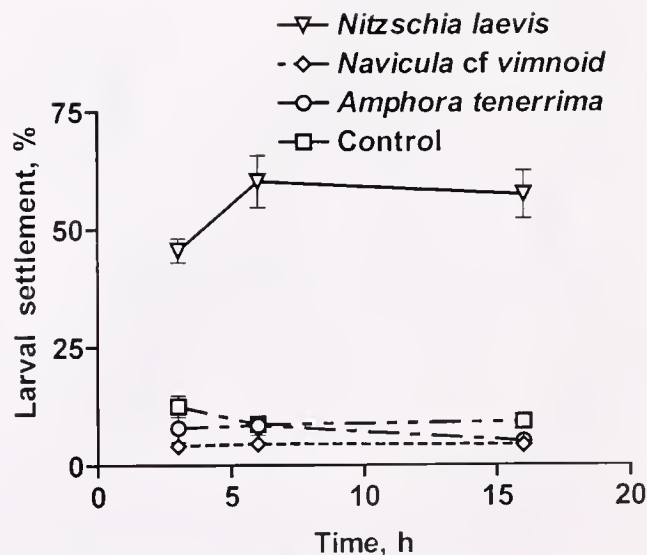


Figure 2. Number of Japanese abalone larvae settled onto patches of 3 diatoms (*Nitzschia laevis*, *Navicula cf. vimnoides* and *Amphora tenerrima*) and a control (no algae) in the course of 16 h. Means (\pm S.E.), $n = 8$.

TABLE 2.

The attraction rank (column A) of each of the examined diatoms (column B). The diatoms were ranked according to their attractiveness to larvae, based on the number of experiments each diatom was offered to abalone larvae (column C), and the number of experiments (with % of experiments from column C in parenthesis) in which larvae significantly (1-w ANOVA, $P < 0.05$) preferred to attach onto that diatom species (column D).

A Attraction Ranking	B Diatom Species	C Number of Experiments	D No. of Experiments with Significant Attraction
1	<i>Nitzschia laevis</i>	31	31 (100%)
2	<i>Navicula cf lenzii</i>	28	23 (82%)
3	<i>Amphora luciae</i>	12	9 (75%)
4	<i>Nitzschia laevis</i> (symbiont clone)	11	7 (64%)
5	<i>Amphora coffeiformis</i>	6	4 (66%)
6	<i>Amphora</i> sp. (1)	6	4 (67%)
7	<i>Navicula cf viminoides</i>	8	4 (50%)
8	<i>Navicula</i> sp. (1)	4	2 (50%)
9	<i>Navicula</i> sp. (2)	2	1 (50%)
10	<i>Navicula salinicula</i>	9	3 (33%)
11	<i>Amphora</i> sp. (2)	7	2 (28%)
12	<i>Amphora</i> sp. (3)	7	2 (28%)
13	<i>Amphora cf tenerrima</i> (1)	9	1 (11%)
14	<i>Amphora</i> sp. (4)	5	1 (20%)
15	<i>Navicula</i> sp. (3)	11	1 (9%)
16	<i>Amphora cf tenerrima</i> (2)	5	0
17	<i>Amphora</i> sp. (6)	5	0

Surprisingly, the settlement on sonicated *N. laevis* was much lower than that on *A. cf tenerrima* (2) and in one experiment it did not differ from the control (no algae) (see Fig. 3).

The temporal dynamics of the settlement process were also different with the sonicated diatoms. When comparing larval settlement onto whole cells and onto sonicated cells in the same petri dish, larvae explored and attached first (after 1.3 h) onto the sonicated cells, and gradually over time moved to settle onto the whole cells (Fig. 4). After 22 hours, larval settlement onto patches of intact cell layers was significantly (1-w ANOVA, $P < 0.001$) higher than onto patches of the sonicated cell suspensions or the control (no algae).

Settlement of Abalone Larvae on Boiled Sonicated Diatom Cells

Larval preference for settlement onto sonicated cells of three of the four diatom species tested was not significantly affected (1-w ANOVA, $P > 0.05$) by boiling them (Fig. 5). Only with sonicated *Amphora luciae* cells, did the larval preference significantly drop (1-w ANOVA, $P < 0.001$) after boiling, even though it still remained significantly above the preference (1-w ANOVA, $P = 0.001$) for the control (no algae) (Fig. 5).

Settlement of Japanese Abalone Larvae on Agar Plugs Soaked with Algal Growing Medium and Amino Acid Solutions

The abalone larvae settlement on agar plugs soaked with algal growing medium, in which attractive diatoms had been grown, did

not produce significant results (1-w ANOVA, $P > 0.05$) (Table 4). Settlement on agar plugs soaked with 10 mM of aspartic acid was consistently much (13 times) higher than on the control (no algae) plug ($P < 0.001$ Table 5). However, settlement on 16 other amino acids tested, singly and in mixtures, could not be distinguished (1-w ANOVA, $P > 0.05$) from the control (see Table 5).

The Effect of Light on the Settlement of Abalone Larvae

There was no significant effect of light (1-w ANOVA, $P > 0.05$) on the settlement of larvae (Fig. 6).

DISCUSSION

Post larvae of abalone are able to distinguish between diatom species, feeding on some and avoiding others (Norman-Boudreau et al. 1986, Matthews & Cook 1995, Siqueiros-Beltrones & Voltolina 2000). This behavior is similar to that which has been observed in many meiofauna that were attracted to and retained by patches of micro-algae that they selected (Lee et al. 1977). In this context it was not surprising that the larvae of *Haliotis discus hamai* in our experiment preferred to selectively attach and settle on some of the diatom patches offered to them but not on others. The chemosensory nature of the process, by which swimming abalone larvae select where to attach and where to settle, has been confirmed by the success of our novel "cafeteria" method by which "good" diatoms were selected. This method was based on the ability of abalone to sense and select the site of settlement and source of food according to several proposed environmental cues (Morse 1985, Morse 1992, Baxter & Morse 1992, Searcy-Bernal et al. 1992, Fleming 1995, Bryan & Qian 1998, Roberts 2001).

Not many diatoms are known to be consistently suitable for abalone larval recruitment (Roberts 2001). Most of the diatoms we tested were indeed unsuitable (as has also been reported in Daume et al. 1999), even though they formed flat colonies, which had been reported as suitable for abalone larval settlement (Kawamura 1996). Nevertheless we have found three highly attractive diatoms: *Nitzschia laevis*, which was by far the best, followed by *Navicula lenzii* and *Amphora luciae*.

In accordance with the results of the study by Morse and Morse (1984) on *Haliotis rufescens* larvae, the cue that allows the Japanese abalone larvae to select one diatom over another for settlement was not visual or physical, but probably chemical. Apparently, the larvae swim towards settlement-inducing substances that are released by the diatoms and descend around them (Searcy-Bernal et al. 1992). If the diatoms are nutritious, the larvae attach and metamorphose; otherwise they ascend and swim on.

Sonication that causes release of cellular material into the surrounding water enhanced *Amphora cf tenerrima* (2) attractiveness for larval settlement, whereas intact cells were not found attractive. Morse and Morse (1984) obtained similar results in their study with *Haliotis rufescens* by surface abrasion of the seaweed *Porphyra* sp. Evidently, sonication or abrasion of "non-attractive" algae releases or speeds up the release of attractive substances that are tightly contained naturally in their intact cells.

The high preference of the abalone larvae to descend onto broken (sonicated) cells, in contrast to the lack of descent onto plugs soaked with algal growth medium, implies that attachment and settlement are concentration dependent and may explain the

TABLE 3.

The settlement preference of Japanese abalone larvae for different diatoms in 8 typical positive comparisons, calculated for species that had been found significantly (1-w ANOVA, $P < 0.05$) attractive in 50% or more of the experiments in which they had been offered. Means include all experiments with the specific combination. Each combination tested attraction toward a control of Bacto-agar plus without algae, and a reference plug with a highly attractive alga (*Nitzschia laevis* or *Navicula lenzii*).

Combination No.	Diatom Species	% Attraction \pm SE (*)	Combination No.	Diatom Species	% Attraction \pm SE (*)
1	<i>Nitzschia laevis</i>	38.4 \pm 3.2 (a)	5	<i>N. laevis</i>	41.8 \pm 5.0 (a)
	<i>Navicula cf lenzii</i>	27.5 \pm 2.3 (b)		<i>Amphora</i> sp. (1)	18.6 \pm 2.2 (b)
	Control	12.6 \pm 1.6 (c)		Control	13.6 \pm 1.9 (b)
2	<i>Navicula cf lenzii</i>	32.8 \pm 2.9 (a)	6	<i>N. laevis</i>	61.4 \pm 6.0 (a)
	<i>Amphora luciae</i>	26.6 \pm 2.5 (a)		<i>Navicula cf vimnoides</i>	12.4 \pm 1.1 (b)
	Control	9.8 \pm 1.1 (b)		Control	13.5 \pm 3.4 (b)
3	<i>Navicula cf lenzii</i>	28.2 \pm 3.5 (a)	7	<i>Navicula cf lenzii</i>	35.9 \pm 3.4 (a)
	<i>Nitzschia laevis</i> (symbiont clone)	24.2 \pm 4.2 (a)		<i>Navicula</i> sp. (1)	22.5 \pm 2.7 (b)
	Control	10.4 \pm 1.8 (b)		Control	14.8 \pm 2.6 (b)
4	<i>Navicula cf lenzii</i>	37.0 \pm 2.3 (a)	8	<i>N. laevis</i>	45.7 \pm 2.0 (a)
	<i>Amphora coffeiformis</i>	22.4 \pm 2.4 (b)		<i>Amphora cf tenerrima</i> (2)	15.4 \pm 6.4 (b)
	Control	11.6 \pm 1.2 (c)		Control	19.2 \pm 4.6 (b)

* Data with the same letter indicate treatments that are not significantly different from each other (ANOVA, Duncan's multiple range test, $P > 0.05$).

link between density and age of an algal layer and its suitability for settlement, as reported in previous studies (Kawamura & Kikuchi 1992, Daume et al. 1999).

The process of larval swimming, descent on several sites and attachment to a certain diatom patch as seen in our work and noted earlier by others (Seki 1997, Bryan & Qian 1998) is a serial cue process. Our data and observations confirm that in the process of substratum exploration, the descent of larvae to a certain spot usually depends on a cue, different in kind or in quantity from the cue for attachment and metamorphosis. The attachment is most probably induced by the mutual and perhaps synergistic action of several cues (Trapido-Rosental & Morse 1986). If larvae are given a choice, attachment and metamorphosis will most probably depend on specific attractive and repellent compounds (see below), in addition to the obvious cue of the nutritive value of the patch. Repellent or harmful substances can reduce descent or elicit ascent of the larvae (Seki 1997, Bryan & Qian 1998, Roberts 2001). This can explain why larvae that quickly descended in large numbers on a sonicated diatom suspension patch, eventually moved on and settled on patches of intact algae, apparently nutritive even though comparatively less attractive for settlement. Seki and Taniguchi

(1996) and Takami et al. (1997) found similar results with mucus induction: abalone larvae settled preferentially on trails of apparently more nutritive mucus, secreted by juvenile or adult abalone during grazing of diatoms, than on mucus secreted by adults during crawling without feeding. Aspartic acid strongly induced descent and attachment of swimming larval abalone. It was also the only free amino acid traced in the growth media of two diatoms, the attractive *Nitzschia laevis* and the unattractive *Amphora cf tenerrima*.

TABLE 4.
Larval settlement, tested toward agar layers made with liquid media in which diatoms had been grown, in comparison to whole cell diatom layers on agar. Mean number of settled larvae \pm SE; $n = 10$; data with the same letter indicate treatments that are not significantly different (ANOVA, Duncan's multiple range test, $P > 0.05$).

Diatom Species	No. of Larvae Settled on Agar Plugs with Whole Cells	No. of Larvae Settled on Agar Plugs with Media
<i>Nitzschia laevis</i>	44 \pm 3.6 a	38 \pm 2.9 a
<i>Amphora cf tenerrima</i> (2)	28 \pm 3.2 b	29 \pm 3.4 a
Control	28 \pm 3 b	33 \pm 2.8 a

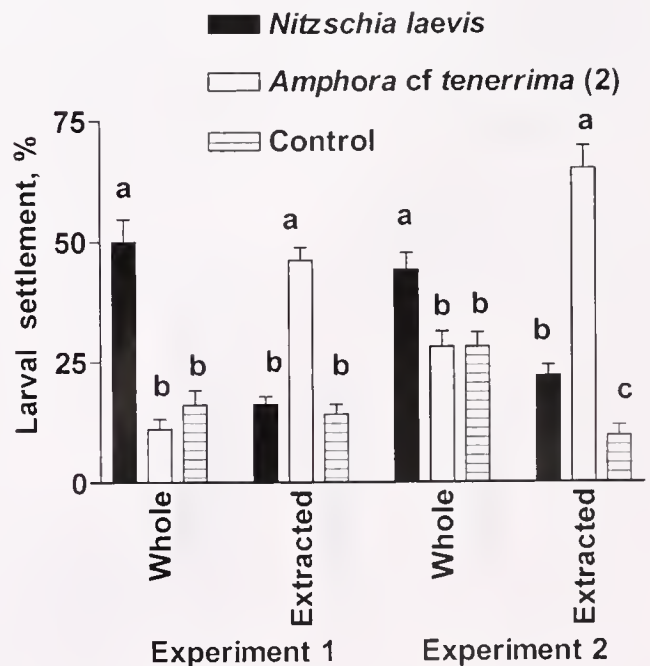


Figure 3. Settlement of Japanese abalone larvae on agar plugs covered by whole diatom cells in comparison with agar plugs covered by sonicated cells (mixed in agar plugs) of the same diatoms in different dishes. Means (\pm S.E.), $n = 10$; data with the same letter indicate treatments that are not significantly different (ANOVA, Duncan's multiple range test, $P > 0.05$).

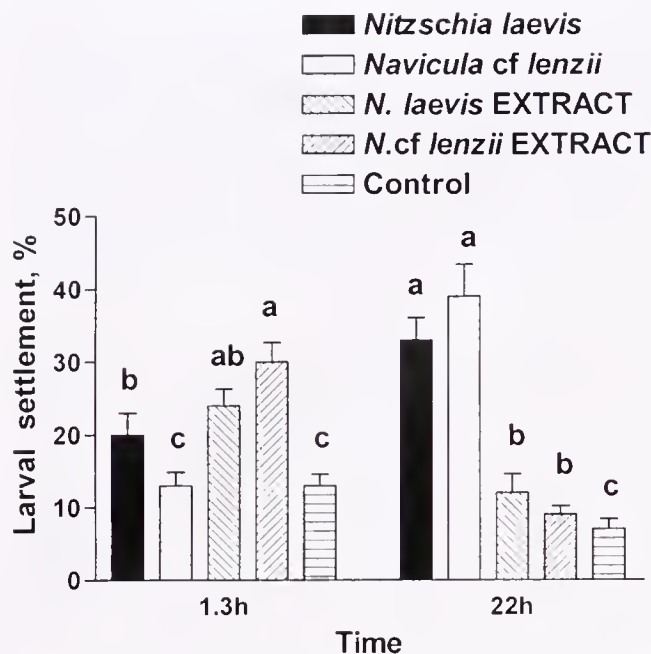


Figure 4. Settlement of Japanese abalone larvae on layers of whole diatom cells in comparison with sonicated diatom cells (mixed in agar plugs) in the same dish. Means (\pm S.E.), $n = 10$; data with the same letter indicate treatments that are not significantly different (ANOVA, Duncan's multiple range test, $P > 0.05$).

errima (2). However, the concentrations of aspartic acid in these media were two orders of magnitude lower than inside algal cells, and three orders of magnitude lower than the level needed to induce larval attachment in our experiments. Aspartic acid might therefore be only one of several factors concurrently involved in larval settlement induction. The rate of the chemical release to the water by the intact cells and the sharpness of the resulting chemical concentration gradients around them might also be important aspects, as suggested by the inability of the other diatom (*A. cf*

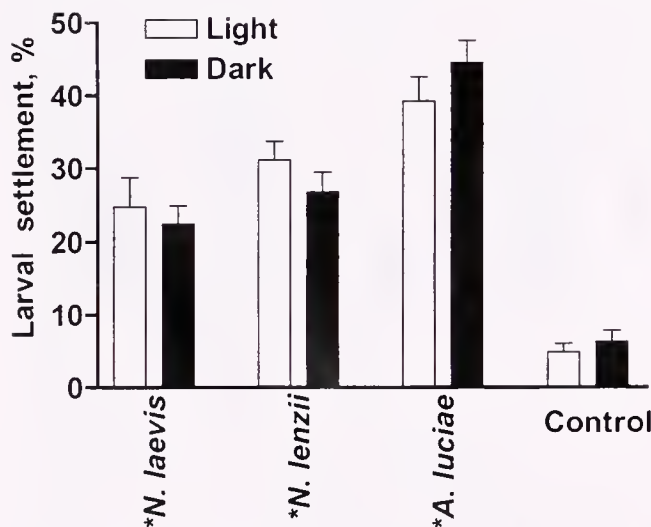


Figure 6. Settlement of Japanese abalone larvae on sonicated cells (mixed in agar plugs) of different diatom species, in dark and light experiments. Means (\pm S.E.), $n = 10$; **N. laevis* = *Nitzschia laevis*; *N. lenzii* = *Navicula cf lenzii*; *A. luciae* = *Amphora luciae*.

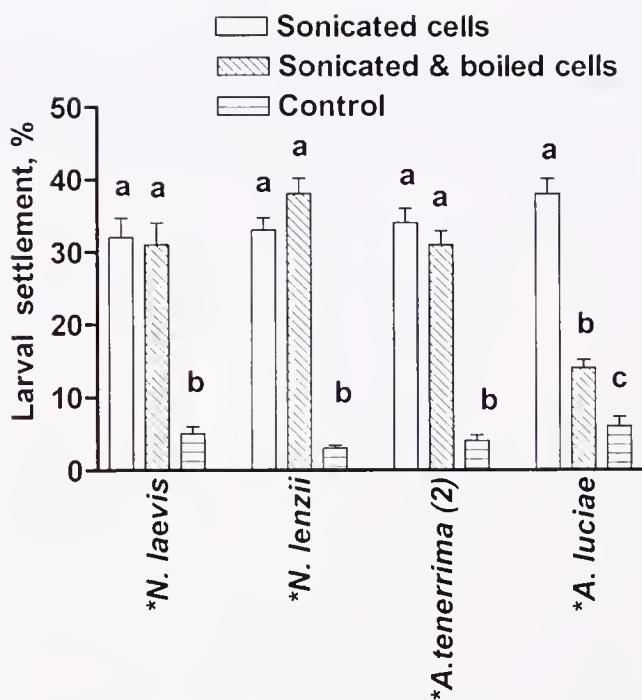


Figure 5. Settlement of Japanese abalone larvae on boiled sonicated cells (mixed in agar plugs) in comparison with the same sonicated cells, un-boiled (mixed in agar plugs), in the same dish. Means (\pm S.E.), $n = 10$; data with the same letter indicate treatments that are not significantly different (ANOVA, Duncan's multiple range test, $P > 0.05$); **N. laevis* = *Nitzschia laevis*; *A. tenerrima* (2) = *Amphora cf tenerrima* (2); *N. lenzii* = *Navicula cf lenzii*; *A. luciae* = *Amphora luciae*.

tenerrima (2)) to attract larval descent with this amino acid in its surroundings. Chemical gradients that are sufficient for chemosensory attraction of larvae can be maintained even in turbulent water, let alone still water as in these experiments (Turner et al. 1994).

Complex native proteins are unlikely to be responsible for the attractiveness of a diatom to larval settlement, because heat denaturation of proteins in sonicated diatom suspensions hardly affected the results of the settlement experiments. Still, small peptides or free amino acids, not affected by heat-denaturation and therefore not excluded by the boiling procedure, have been proposed as possible as chemical cues for abalone larval settlement (Morse & Morse 1984, Morse & Morse 1988, Morse 1985).

Layers made of certain diatoms, such as the three species selected in the research reported here, but not many others, apparently provide the swimming larvae with the whole battery of cues they

TABLE 5.

Larval settlement tested towards solutions (10 mM final concentration) of four amino acids soaked into agar plugs.

Amino Acid	Attracted Larvae (%)
Alanine	4 \pm 1
Proline	3 \pm 1
Leucine	7 \pm 2
Aspartic acid	81 \pm 4
Control	6 \pm 3

Mean \pm S.E.; $n = 10$.

need for descent, attachment, metamorphosis, and nutritious feeding. Layers of diatoms such as those selected here improve attachment and settlement and supply proper nutrition for early post larval stages. These qualities greatly improve survival and growth of the abalone early post larvae (Slattery 1992, Searcy-Bernal et al. 1992, Matthews & Cook 1995, Daume et al. 1999, Roberts 2001). This understanding helps explain the interrelationship between

diatom biodiversity and the recruitment of animals, such as abalone, both in natural settings and in aquaculture.

ACKNOWLEDGMENTS

The authors thank H. Krogliak for providing technical help, M. Ben-Shaprut for the editorial assistance, and all the reviewers for their constructive comments.

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TAXONOMIC APPENDIX

The isolates used in this study have been identified to the closest described taxa. We recognize that species concept in dia-

toms has been interpreted in many ways. Some have very rigid "morpho-species" concepts that allow very little deviation from the type specimen (e.g., Clavero et al. 2000) whereas others (e.g., Lee et al. 2000) have looked at a wide variance in overlapping populations obtained from different field collections. We acknowledge the problem, as it relates to our isolates, but do not wish to resolve this conundrum here.

Some of the diatoms were easier to identify than were others. Four species were problematic. We chose to identify the organism #3 in Table 1 as *Navicula* cf *vinuoides* Giffen. The clone culture we used fit the original description and illustrations of the species.

Navicula cf *lenzii* Hustedt 1945 was described as a freshwater species. In his 1961 monograph (see Literature Cited), Hustedt illustrated and described the variations he considered as belonging to this taxon. Although we would be skeptical to find a freshwater species in a eutrophic marine environment, the species with which we worked is more closely related to this taxon than to any other.

Organisms numbered 5 and 6 in Table 1 were referred to *Amphora* cf *tenerrima* Aleem and Hustedt. They were morphologically distinct from each other in SEM micrographs, but resembled the original light microscopic description of the species by Aleem and Hustedt.

LETHAL LEVELS OF DISSOLVED OXYGEN FOR *HALIOTIS DIVERSICOLOR SUPERTEXTA* AT DIFFERENT SALINITY LEVELS

SHA-YEN CHENG,¹ YIN-HUNG CHANG² AND JIANN-CHU CHEN^{2,*}

¹Department of Environmental Biology and Fisheries Science and ²Department of Aquaculture, College of Life and Resource Sciences, National Taiwan Ocean University, Keelung 202, Taiwan, Republic of China

ABSTRACT The abalone *Haliotis diversicolor supertexta* juveniles (shell length 3.51 ± 1.10 cm) were exposed to different concentrations of dissolved oxygen (DO) using the static renewal method at different salinity levels of 25‰ (g kg⁻¹) and 35‰ at pH 8.04 and 22°C. The 72-, 84-, and 96-h LC₅₀ values of DO on *H. diversicolor supertexta* juveniles were 1.56, 1.76, 2.02 mg L⁻¹ at 25‰ and 1.43, 1.65, 1.83 mg L⁻¹ at 35‰, respectively. As the salinity decreased from 35‰ to 25‰, susceptibility to DO increased by 107%, 107%, and 110% after 72-, 84-, and 96-h exposure, respectively. The relationship between LC₅₀ of DO (mg L⁻¹), salinity (S) and exposure time (T) in the range 72–96 h is as follows:

$$LC_{50} = 0.541 - 0.012S + 0.018T \quad (R^2 = 0.637)$$

KEY WORDS: dissolved oxygen, *Haliotis diversicolor supertexta*, salinity, tolerance

INTRODUCTION

Taiwan abalone (also known as small abalone) *Haliotis diversicolor supertexta* is commercially important in Taiwan as well as in the southeast coast of mainland China as a primary cultured species. For growth, Chen (1984) has observed that salinity in the range 30–35‰ and temperature in the range 24–30°C are the optimal levels. Culture of *H. diversicolor supertexta* has expanded greatly since 1986 due to successful artificial propagation and development of multiple-tier basket systems in grow-out farms (Yang and Ting 1986, Chen and Lee 1999).

Since late 2000, farmers have experienced mass mortality of abalone reared in the multiple-tier basket systems in the grow-out ponds and settlement failure of spat larvae in the nursery ponds. The bacteria *Vibrio alginolyticus* and *V. parahaemolyticus* isolated from the hemolymph of moribund abalone have been documented to cause vibriosis leading to death of abalone in warmer water temperatures (Liu et al. 2000, Lee et al. 2001). As a result, the farmed production of abalone in Taiwan declined from 2497 tons in 2001 to 1089 tons in 2003.

Yang and Ting (1989) reported that dissolved oxygen (DO) should be maintained higher than 5 mg L⁻¹ for the optimal growth of *H. diversicolor supertexta*. They also reported that the oxygen consumption of this species maintained at 32‰ and 30°C was 1.3 times that maintained at 32‰ and 25°C. Fallu (1991) reported that abalones require DO greater than about 3–4 mg L⁻¹. It is very common that the pond water in multiple-tier basket systems may become hypoxic or even anoxic due to respiration of animals and decomposition of accumulated organic matter such as unconsumed food and feces. Therefore, concentrations of DO in pond water and its lethal levels on abalone are of primary concern.

Lethal levels of DO have been reported for a number species of penaeid shrimps including kuruma shrimp *Marsupenaeus japonicus* (Egusa 1961, Tournier 1972), caramote prawn *Melicerus kerathurus* (Tournier 1972), tiger shrimp *Penaeus monodon* (Liao & Huang 1975, Allan & Maguire 1991), southern white shrimp *Litopenaeus setiferus* (MacKay 1974), and northern brown shrimp

Farfantepenaeus aztecus (Martínez et al. 1998). Concerning the mollusks, Miller et al. (2002) reported that the 96-h LC₉₀ (90% of animal killed after 96 h) of DO on the Atlantic surf-clam *Spisula solidissima* was 0.3 mg L⁻¹. Tamai (1993) reported that the semelid small bivalve *Theora fragilis* survived DO as low as 1.3–1.4 mg L⁻¹ at least for four days. Temperature has been reported to affect the tolerance of low DO on zebra mussel *Dreissena polymorpha* and Asian clam *Corbicula fluminea* (Johnson & McMahon 1998). However, with a few minutes of literature searching, we found no information available on the lethal level of DO on marine invertebrates at different salinity levels. The purpose of the current study is to estimate the lethal level of DO on *H. diversicolor supertexta* at 25‰ and 35‰.

MATERIALS AND METHODS

Seawater and Test Solution

Seawater pumped from the Keelung coast adjacent to the National Taiwan Ocean University was adjusted to 35‰ (g kg⁻¹) and 25‰, with municipal water dechlorinated with sodium thiosulfate, and then filtered through a gravel and sand bed by air-lifting and aerating for three days before use. The concentration of DO in water was regulated with nitrogen gas that was bubbled into the water to produce a series of DO that ranged from 6.87 to 7.46, 5.12 to 5.88, 3.19 to 3.80, 2.19 to 2.77, and 1.24 to 1.62 mg L⁻¹ with an average of 7.11, 5.53, 3.45, 2.47, and 1.44 mg L⁻¹, respectively. These served as test solutions.

Animals

H. diversicolor supertexta were obtained from a commercial abalone farm in Ilan, Taiwan, and acclimated in the laboratory with salinity of 35‰ for two weeks prior to experimentation. The abalones were divided randomly into two groups in the same salinity and then adjusted 2‰ each day to reach two different salinity levels of 25‰ and 35‰. After the abalones reached the expected salinity levels, they were reared for one more week. The mean (±SD) wet body weight and shell length was 4.12 ± 0.9 g and 3.51 ± 1.10 cm, respectively, with no significant difference among treatments.

*Corresponding author. Fax: 886-2-2462-0295; E-mail: jcchen@mail.ntou.edu.tw

Lethal Effect of Dissolved Oxygen

Short-term LC_{50} (median lethal concentration) toxicity tests were carried out according to the methods described by the American Public Health Association et al. (1985). Abalones were sampled randomly from the holding tanks and transferred to test solutions. Bioassay experiments to establish tolerance limits were conducted in 20-L glass flasks containing 10-L test solutions. Each tank contained 10 test abalones. Each test solution was renewed daily, in accordance with the static renewal method for toxicity tests (Buikema et al. 1982). There were three replicates for each test solution with a total number of 30 abalones (10 per replicate for each test solution). During the experiment, abalones were fed *Gracilaria tenuistipitata* daily. Water temperature was maintained at $22 \pm 1^\circ\text{C}$ and pH ranged from 7.87 to 8.12 with an average of 8.04.

Observations were usually made at 6-h intervals up to 168 h. Abalone that failed to respond to tactile stimulation when touched with a glass rod were defined as dead. The LT_{50} (median lethal time, hours required to kill half the population) was determined. Dead abalones were removed daily. The concentration response of test organisms was determined for LC_{50} (median lethal concentration) value of dissolved oxygen and their 95% confidence limits with a computer program (Trevors and Lusty 1985) based on a method described by Hubert (1980). The estimated probit line and the results of a chi-square (χ^2) test for goodness of fit were computed.

Statistical Analysis

The LC_{50} s of dissolved oxygen (DO) were subjected to one-way analysis of variance (ANOVA) followed by Duncan multiple range test (Duncan 1955). Relationship between survival, salinity, DO concentration, and exposure time (hours) and the relationship between LC_{50} of DO, salinity, and exposure time were tested using the General Linear Model procedure and Regression Procedure, version 6.03, Statistical Analysis System (SAS) computer software (SAS 1998). All statistical significance tests were at the $P < 0.05$ level.

RESULTS

All abalones survived in the test solutions at 5.53 and 7.11 mg L^{-1} DO at all salinity levels after 168 h exposure. No abalone died in 1.44 and 2.47 mg L^{-1} DO after 36 h at 25‰. However, all abalones exposed to 1.44 mg L^{-1} DO at 25‰ died after 108 h. No abalone died in 1.44 and 2.47 mg L^{-1} after 24 h and 48 h, respectively, at 35‰. However, all abalones exposed to 1.44 mg L^{-1} DO at 35‰ died after 108 h (Fig. 1). There was a significant effect of DO, salinity, and exposure time on survival. There was also a significant interaction between the effects of salinity and DO and a significant interaction between the effects of DO concentration and exposure time on survival. There was no significant interaction between the effects of salinity and exposure time on survival. In salinity levels of 25–35‰, the relationship between survival (%), DO concentration (C), salinity (S), exposure time (T), and the interactions between salinity and DO concentration (SC), DO concentration and exposure time (CT) is as follows:

$$\text{Survival (\%)} = 123.125 + 0.340S - 1.439T - 7.340C - 0.054SC + 0.418CT \quad (R^2 = 0.858)$$

For the abalones exposed to 1.44 mg L^{-1} DO at 25‰ and 35‰, there was a significant effect of salinity and exposure time on

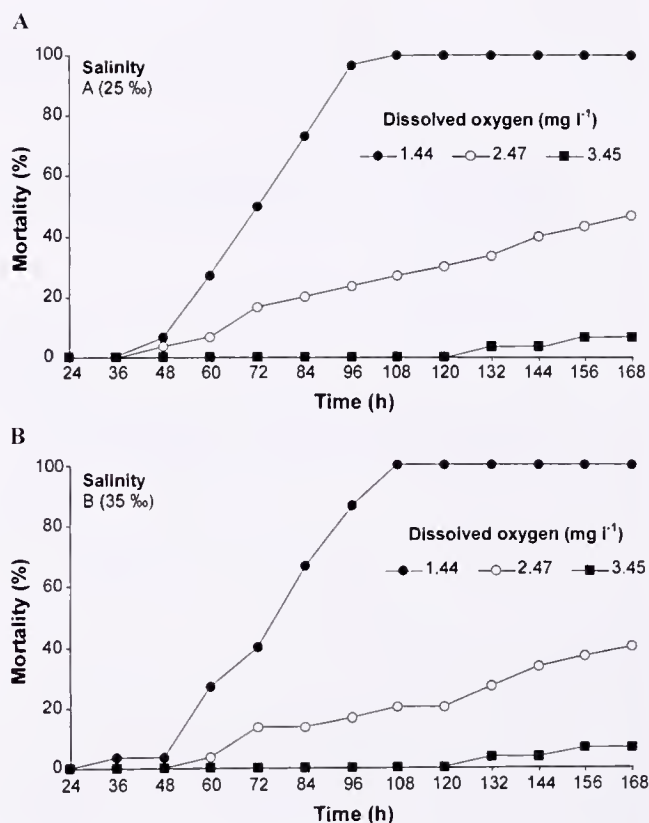


Figure 1. The percentage mortality of *Haliotis diversicolor supertexta* juveniles exposed to different dissolved oxygen consumption at (A) 25‰ (A) and (B) 35‰.

survival. However, there was no significant interaction between the effects of salinity and exposure time on survival. In 1.44 mg L^{-1} DO, the relationship between survival, S, and T is as follows:

$$\text{Survival (\%)} = 116.210 + 0.138S - 0.879T \quad (R^2 = 0.858).$$

For the abalones exposed to 2.47 mg L^{-1} DO at 25‰ and 35‰, there was a significant effect of salinity and exposure time on survival. However, there was no significant interaction between the effects of salinity and exposure time on survival. In 2.47 mg L^{-1} DO, the relationship between survival, S, and T is as follows:

$$\text{Survival (\%)} = 97.501 + 0.460S - 0.319T \quad (R^2 = 0.847).$$

In 25‰, the LT_{50} (median lethal time, hours) values of *H. diversicolor supertexta* juveniles exposed to 2.47 and 1.44 mg L^{-1} DO was 179 and 72 h, respectively, whereas in 35‰, the LT_{50} of abalone exposed to 2.47 and 1.44 mg L^{-1} DO was 204 and 75 h, respectively. The probit of mortality for *H. diversicolor supertexta* juveniles exposed to DO was linearly related to log DO at various exposure times (Table 1). A χ^2 test indicated that values of chi were less than the values in those tables, suggesting that the assumed lines were satisfactory (Trevors & Lusty, 1985).

The LC_{50} of DO values (and their 95% confidence limits) at different exposure periods for *H. diversicolor supertexta* juveniles are shown in Figure 2. At 72, 84, and 96 h, the LC_{50} values of DO were 1.56, 1.76, and 2.02 mg L^{-1} at 25‰ and 1.43, 1.65, and 1.83 mg L^{-1} at 35‰. Resistance of *H. diversicolor supertexta* juveniles to DO was 9.0%, 6.7%, and 10.3% less at 25‰ than that at 35‰ after 72, 84, and 96 h exposure. The LC_{50} of DO was inversely

TABLE 1.

Relationship between probit of mortality (Y) and log DO as mg L⁻¹ (X) at various exposure times at 25‰ and 35‰ for *H. diversicolor supertexta* juveniles.

Time (h)	$Y = a + bX$	n^*	X^2 †	χ^2 Calculated	χ^2 df	0.95
25‰						
72	$Y = 15.232 - 8.572X$	3	0.868	13.278	1	3.747
78	$Y = 15.861 - 8.996X$	3	0.890	12.840	1	3.747
84	$Y = 17.752 - 10.232X$	3	0.916	10.645	1	3.747
90	$Y = 19.178 - 11.193X$	3	0.945	7.745	1	3.747
96	$Y = 22.480 - 13.394X$	3	0.976	4.461	1	3.747
35‰						
72	$Y = 13.921 - 7.729X$	3	0.857	11.297	1	3.747
78	$Y = 14.951 - 8.424X$	3	0.896	9.945	1	3.747
84	$Y = 16.628 - 9.559X$	3	0.940	6.297	1	3.747
90	$Y = 18.376 - 19.736X$	3	0.969	4.033	1	3.747
96	$Y = 19.321 - 11.339X$	3	0.968	4.373	1	3.747

* Number of concentration for calculation.

† Coefficient of determination.

related with salinity level on *H. diversicolor supertexta* juveniles. The LC₅₀ values of DO at 35‰ were significantly lower than those at 25‰ on *H. diversicolor supertexta* juveniles.

Statistical analysis indicated that there was a significant effect of salinity on LC₅₀ and exposure time on LC₅₀. However, there was no significant interaction between the effects of salinity and exposure time on LC₅₀. The relationship between LC₅₀ of DO (mg L⁻¹), S, and T is as follows:

$$LC_{50} = 0.541 - 0.012S + 0.018T \quad (R^2 = 0.637).$$

DISCUSSION

The sublethal concentrations of DO for selected marine and estuarine teleosts and invertebrates have been studied (Table 2).

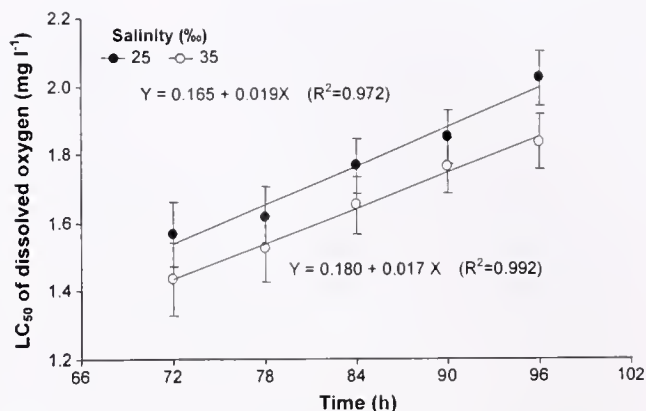


Figure 2. LC₅₀ (median lethal concentration) of dissolved oxygen and its 95% confidence limits to *Haliotis diversicolor supertexta* juveniles exposed to different concentrations of dissolved oxygen at 25‰ and 35‰.

Reported 96-h LC₅₀ of DO on juvenile teleost ranged from 0.6 mg L⁻¹ for northern sea robin *Prionotus carolinus* to 1.6 mg L⁻¹ for summer flounder *Paralichthys dentatus* and striped bass *Morone saxatilis* (Miller et al. 2002). Reported 96-h LC₅₀ of DO on juvenile decapod crustaceans ranged from 0.7 mg L⁻¹ for daggerblade grass shrimp *Palaemonetes pugio* to 2.9–4.4 mg L⁻¹ for northern brown shrimp *F. aztecus* (Stickle et al. 1989, Allan and Maguire 1991, Miller et al. 2002). The 96-h and 168-h LC₅₀ of DO on blue crab *Callinectes sapidus* was 2.5–4.1 mg L⁻¹ and 2.3 mg L⁻¹, respectively (Stickle et al. 1989, Das and Stickle 1993). Reported 96-h LC₅₀ of DO on juvenile mollusc was 0.5 mg L⁻¹ for Atlantic surfclam *Spisula solidissima* at 28–32‰ (Miller et al. 2002) and 2.02 mg L⁻¹ and 1.83 mg L⁻¹ for *H. diversicolor supertexta* at 25‰ and 35‰, respectively, in the current study. These facts indicated that the abalone *H. diversicolor supertexta* was less tolerant to low DO than *P. monodon* and *S. solidissima* but was more tolerant to low DO than *F. aztecus* and *C. sapidus*. Liao and Huang

TABLE 2.

The LC₅₀s (median lethal concentrations) of dissolved oxygen (DO) on several species of teleosts, decapod crustaceans, and mollusc juveniles.

Species	Salinity (‰)	LC ₅₀ (mg L ⁻¹)	Reference
Teleosts			
<i>Prionotus carolinus</i>	28–32	96-h 0.6	Miller et al. (2002)
<i>Paralichthys dentatus</i>	28–32	96-h 1.6	Miller et al. (2002)
Decapod crustaceans			
<i>Americamysis bahia</i>	28–32	96-h 1.2	Miller et al. (2002)
<i>Homarus americanus</i>	28–32	96-h 1.0	Miller et al. (2002)
<i>Palaemonetes vulgaris</i>	28–32	96-h 1.0	Miller et al. (2002)
<i>Palaemonetes pugio</i>	28–32	96-h 0.7	Miller et al. (2002)
<i>Crangon septemspinosa</i>	28–32	96-h 1.0	Miller et al. (2002)
<i>Cherax tenuimanus</i>	Freshwater	24-h 0.7	Morrissey et al. (1984)
<i>Penaeus monodon</i>	32–35	96-h 0.9	Allan and Maguire (1991)
<i>Farfantepenaeus aztecus</i>	35	96-h 2.9–4.4	Stickle et al. (1989)
<i>Litopenaeus setiferus</i>	15	72-h 1.16	Martínez et al. (1998)
<i>L. setiferus</i>	38	72-h 1.86	Martínez et al. (1998)
<i>Callinectes sapidus</i>	35	96-h 2.5–4.1	Stickle et al. (1989)
<i>C. sapidus</i>	35	168-h 2.3	Das and Stickle (1993)
Molluscs			
<i>Spisula solidissima</i>	28–32	96-h 0.5	Miller et al. (2002)
<i>Haliotis diversicolor supertexta</i>	25	96-h 2.02	Current study
<i>Haliotis diversicolor supertexta</i>	35	96-h 1.83	Current study

(1975) reported that the lethal level of DO was 0.2–0.3 mg L⁻¹ for *P. monodon*. Allan and Maguire (1991) reported that all *P. monodon* juveniles exposed to 0.3 mg L⁻¹ were killed within 12 h. The current study indicated that *H. diversicolor supertexta* exposed to 1.44 mg L⁻¹ DO were killed within 108 h.

In addition to lethality, growth of *H. diversicolor supertexta* may be affected by low DO. Sediman and Lawrence (1985) reported that the level of DO at 1.2 mg L⁻¹ reduces significantly the growth, and the levels of DO higher than 2 mg L⁻¹ do not affect the growth of *P. monodon* and whiteleg shrimp *Litopenaeus vannamei*. Yang and Ting (1989) reported that DO should be maintained higher than 5 mg L⁻¹ for the optimal growth of *H. diversicolor supertexta*. However, we do not know the level of DO that affects the growth of abalone.

Most of the previous papers documented LC₅₀ values of DO for teleosts and invertebrates at one salinity level only. Abalones are generally cultured intensively in outdoor semi-static pond water or an indoor multiple-tier basket system with salinity varying from 25‰ to 35‰ (Chen and Lee 1999). Lin and Chen (2001) reported that the toxicity of ammonia to *L. vannamei* increased by 104% to 112% as salinity decreased from 35‰ to 25‰. Lin and Chen (2003) reported that the toxicity of nitrite to *L. vannamei* juveniles increased by 161% to 197% as salinity decreased from 35‰ to 25‰. Tsai and Chen (2002) reported that the toxicity of nitrate to *P. monodon* increased by 128% to 147% as salinity decreased from 35‰ to 25‰. Martínez et al. (1998) reported that the lethality of low DO to *L. setiferus* increased by 160% as salinity decreased from 38‰ to 15‰. The current study indicated that the lethality of low DO on *H. diversicolor supertexta* increased by 105% to 111% as salinity decreased from 35‰ to 25‰. Therefore, abalone and shrimp farmers should note the toxicity differences of ammonia, nitrite, nitrate, and low DO at different salinity levels when marking pond management.

In ponds, DO increases during the daytime due to the photosynthesis of phytoplankton and macrophytes and decreases during the nighttime due to the respiration of both plants and animals. It is not surprising that DO depletion may occur in abalone farms, especially in multiple-tier basket systems, due to poor water cir-

ulation. Species of the bacterial *Vibrio* genus are ubiquitous in the marine and estuarine environments. Disease outbreaks have been reported to be associated with increases in the *Vibrio* population of the cultured water (Sung et al. 1999). Challenging *H. diversicolor supertexta* with *V. parahaemolyticus* at 2×10^4 cfu abalone⁻¹ and placed in 35‰ water with different concentrations of DO, Cheng et al. (2004) observed that 72-h and 96-h LC₅₀ of DO was 4.71 and 5.17 mg L⁻¹, respectively. They also reported that the abalone exposed to DO at 3.57 mg L⁻¹ or lower showed decreases in hemocyte count, release of superoxide anion, and phagocytic activity and clearance efficiency to *V. parahaemolyticus* indicating depression in immune system of *H. diversicolor supertexta*.

The LC₅₀ of DO to *H. diversicolor supertexta* juveniles increased with exposure time. The tolerance of *H. diversicolor supertexta* juveniles to low DO decreased sharply by 13% and 41% after 84 and 96 h as compared with 72-h LC₅₀ at 25‰, and the tolerance of *H. diversicolor supertexta* juveniles to low DO decreased sharply by 15% and 28% after 84 and 96 h as compared with 72-h LC₅₀ at 35‰. Similar phenomenon was obtained previously in penaeid shrimps. Lin and Chen (2003) reported that the tolerance of *L. vannamei* juveniles to nitrite decreased by 11% and 35‰ after 48 and 96 h as compared with 24-h LC₅₀ at 25‰, and the tolerance of *L. vannamei* juveniles to nitrite decreased by 19% and 38% after 48 and 96 h as compared with 24-h LC₅₀ at 35‰.

In conclusion, the 72 and 96 h LC₅₀ of DO on *H. diversicolor supertexta* is 1.56 and 2.02 mg L⁻¹ at 25‰ and 1.43 and 1.83 mg L⁻¹ at 35‰, respectively. It appears that in the range of 25‰ and 35‰, the relationship between LC₅₀ of DO, salinity, and exposure time are $LC_{50} = 0.541 - 0.012S + 0.018T$ ($R^2 = 0.637$). The study has revealed that low DO concentrations might cause either lethality or depressed immune ability of abalone.

ACKNOWLEDGMENTS

This paper was supported by a grant from the Council of Agriculture (Grant No. 91-No-Ke-2.5.3-Yu-F2), Taiwan, Republic of China. We appreciate Mr. Y. C. Lin for his assistance in the experiment.

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POPULATION CHARACTERISTICS AND CO-OCCURRENCE OF THREE EXPLOITED DECAPODS (*PANULIRUS ARGUS*, *P. GUTTATUS* and *MITHRAX SPINOSISSIMUS*) IN BOCAS DEL TORO, PANAMA

HECTOR M. GUZMAN¹* AND ALEXANDER TEWFIK²

¹Smithsonian Tropical Research Institute, Unit 0948, APO AA 34002, ²Department of Biology, McGill University, 1205 Ave. Dr. Penfield, Montreal, Canada, H3A 1B1

ABSTRACT Spiny lobster populations within the Bocas del Toro archipelago appear to be overexploited. Extensive visual surveys over a wide area of reef habitat down to 20 m in depth indicate mean lobster abundance, sizes, and percent maturity levels for both *Panulirus argus* and *P. guttatus* below other areas in the region. The almost complete absence of egg-bearing female lobsters may have serious implications for local production and recruitment and emphasizes the need for revision of management strategies for these populations, which are critical to the socio-economic wellbeing of local artisanal fishers and their dependent communities. An increase in the minimum carapace length for *P. argus* from 60–88 mm may allow protection for most juveniles. The implementation of a closed season or total fishing ban for lobster may also be considered to rebuild the spawning stock biomass to sustainable levels. The extensive pressure likely being exerted on lobster resources may spill over to co-occurring but poorly studied species, such as the large West Indian spider crab, *Mithrax spinosissimus*. This study presents the first data collected on a wild population of *M. spinosissimus*. More importantly, it illustrates the poor understanding we have of the inter-relationships between co-occurring species and the need to understand communities of populations that should be co-managed for the preservation of resources and biodiversity.

KEY WORDS: spiny lobster, *Panulirus*, spider crab, *Mithrax*, stock assessment, overfishing, Panama, shelter, dens

INTRODUCTION

The shallow water environments of the Panamanian coast provide important habitats for a wide variety of animal populations that support a number of important commercial and subsistence fisheries (Cruz 2000, Castillo & Lessios 2001, Guzman & Guevara 2002, Tewfik & Guzman 2003). Throughout these areas and the wider Caribbean region 3 large decapod crustaceans, *Panulirus argus* (Caribbean spiny lobster), *P. guttatus* (spotted spiny lobster), and *Mithrax spinosissimus* (West Indian spider crab) appear to coexist over reef habitats. Humans, to some lesser or greater extent, have attempted to exploit all three species with a particular focus on *P. argus* (Cochrane & Chakalall 2001). *P. guttatus* may be caught incidentally throughout the region but directed fisheries appear to exist only in Bermuda and Martinique (Evans & Lockwood 1994, Evans & Evans 1995). *M. spinosissimus* appears to have the most restricted use with incidental catches in some areas, namely the Panama Canal area, and limited efforts directed at experimental culture of the species (Wilber & Wilber 1991).

Our knowledge of the biology and ecology of these three organisms seems in approximate proportion to their level of exploitation. All three are nocturnal omnivores with varying diurnal den-dwelling preferences (Hazlett & Rittschof 1975, Lozano-Alvarez & Briones-Fourzan 2001). An extensive literature exists for the large *P. argus* and subsequently most lobster resource management in the Caribbean is based on experience with this species (Briones-Fourzan 1995). *P. argus* undergoes a number of ontogenetic habitat shifts, including the use of reef environments, and is highly migratory (Kanciruk & Herrinkind 1978, Butler & Herrinkind 1997). In contrast, the smaller *P. guttatus* appears to be an obligate, shallow-water reef dweller and is not migratory (Sutcliffe 1953, Sharp et al. 1997). Finally, very little is known about *M. spinosissimus* in the wild with limited observations being conducted in semi-natural and culture environments (Hazlett & Rittschof 1975, Wilber & Wilber 1991).

Given the significant role these decapods likely serve as intermediate consumers in reef and associated habitats and the socio-economic importance they have to local free-diving fishers, operating almost exclusively from small canoes (dugout cayucos), an extensive visual survey was conducted for these three species in the Panamanian archipelago of Bocas del Toro. The following paper is the first report of abundance, population structure, morphology, and spatial distribution of *P. argus* and *P. guttatus* in the study area. This work is also the first documented survey of a wild population of *M. spinosissimus* in the wider Caribbean.

MATERIAL AND METHODS

The survey was conducted over approximately 62,533 hectares of shallow water (<20 m) coral reef habitats in the Bocas del Toro archipelago between April and September 2002 (Fig. 1). A comprehensive description of the sea bottom topography, climate, geology, and reef distribution of the archipelago are available in several other publications (Rodriguez et al. 1993, Greb et al. 1996, Guzman & Guevara 1998). A total of 110 sites were randomly selected and surveyed. Within each site three replicate belt transects (100 × 6 m), randomly placed, were surveyed by two divers (3-meter width each) at four different depth strata (0–5 m, 5–10 m, 10–15 m, 15–20 m) when they occurred.

All individual lobsters encountered during the surveys were measured for carapace length (CL) and tail length (TL) to the nearest millimeter and sex was determined. Carapace length was measured from the base of the rostral horns to the posterior edge of the carapace and tail length was measured from the anterior edge of the tail to the posterior tip of the telson. Sex was determined by the presence of biramous pleopods for females and uniramous pleopods for males (Morgan 1980). The presence of a whole or remnant eggmass on the ventral abdominal segments was used to determine female maturity (Chubb 1994). All observed *M. spinosissimus* had carapace width (maximum distance between lateral margins) measured to the nearest millimeter as well as determination of sex again by the presence of biramous or uniramous pleopods. Maturity of females also was assessed based

*Corresponding author. E-mail: guzmanh@naos.si.edu

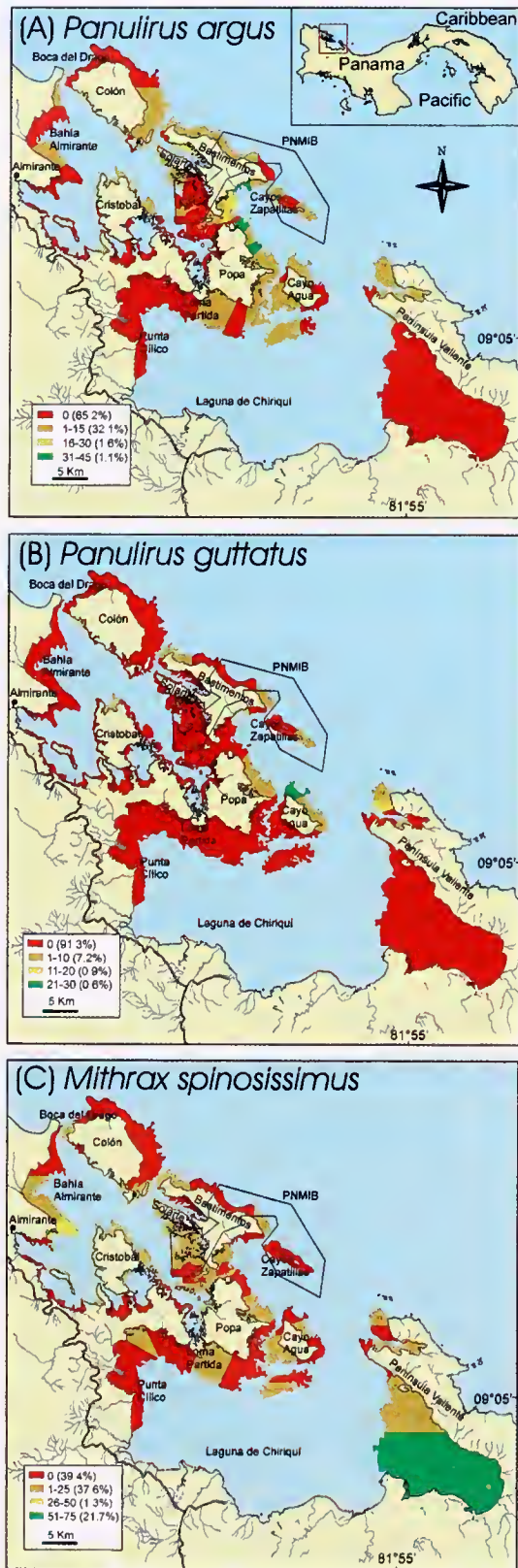


Figure 1. Location of the archipelago of Bocas del Toro in Panama (upper right). Density range distributions (individual ha⁻¹) of *Panulirus argus* (A), *P. guttatus* (B), and *Mithrax spinosissimus* (C) over shallow water reef habitats (<20 m).

on presence of a whole or remnant eggmass (Hernandez-Reyes et al. 2001).

The density distribution of all three species was calculated as mean number of individuals per hectare by site and depth strata. Site data was mapped using Geographical Information System (GIS). A digital classification for the area of study was based on topographic maps at scale 1:50,000. Density data were integrated using the programs MIP V3.1 (Map and Image Processing System) and ArcView V3.0. All data sets for morphologic correlations and abundance were analyzed using SYSTAT V10.2. Parametric techniques were used for correlations and non-parametric (Kruskal-Wallis one-way analysis of variance by ranks and the two-sample Kolmogorov-Smirnov test between pairs) techniques for differences in abundances between species and depth strata given the non-normal distribution of the data due to high numbers of zero values and the poor success of transforming such data.

RESULTS

A total of 496,800 m² (49.6 ha), using 828 transects, were surveyed down to 20 m during the course of the study. A total of 180 *Panulirus argus*, 41 *P. guttatus* and 189 *Mithrax spinosissimus* were found.

Highest densities for *P. argus* (31–45 lobster ha⁻¹) occurred at the North-east side of Popa and the southern edge of Bastimentos (see Fig. 1A). *P. guttatus* was most abundant (21–30 lobster ha⁻¹) at the northern tip of Cayo Agua, the southern edge of Bastimentos, and at the tip of the Peninsula Valiente (see Fig. 1B). All of these areas are associated with complex patch reef habitats. Maximum *M. spinosissimus* densities were found in areas of low visibility south of Peninsula Valiente (51–76 crab ha⁻¹) and at the southern tip of Bahía Almirante (26–50 crab ha⁻¹) (see Fig. 1C).

M. spinosissimus had the highest overall percent occurrence (55.5%) and *P. guttatus* the lowest (13.6%) with *P. argus* appearing at 50.0% of the 110 sites examined. The highest percent occurrence for each species by depth was; *P. argus*, 40.6% at 5–10 m; *P. guttatus*, 10.4% at 10–15 m; and *M. spinosissimus*, 34.0% at 5–10 m. The co-occurrence of species at sites was as follows: the 2 lobster species, 11.8%; *P. argus* and *M. spinosissimus*, 23.6%; *P. guttatus* and *M. spinosissimus*, 7.3%; and all 3 species, 6.4%. At no time was any individual den occupied by more than one species. None of the three species occurred in 22.7% of the sites.

Overall density values (mean individual ha⁻¹ ± SE) for the 3 decapod species, *P. argus*, 3.85 ± 0.66; *P. guttatus*, 0.91 ± 0.30; and *M. spinosissimus*, 4.11 ± 0.87, did vary significantly (Kruskal-Wallis, $H = 43.17$, $df = 2$, $P < 0.0001$). Both *P. argus* (Kolmogorov-Smirnov 2 sample differences, $P < 0.0001$) and *M. spinosissimus* ($P < 0.0001$) overall densities were higher than *P. guttatus*. Analysis of variance (Kruskal-Wallis) by depth revealed significant differences within the 0–5 m strata ($H = 29.55$, $df = 2$, $P < 0.0001$) and the 5–10 m strata ($H = 30.83$, $df = 2$, $P < 0.0001$) between *P. guttatus* and the other two species (Fig. 2). None of the three species individually varied significantly across the four depth strata. However, no *P. guttatus* were found below 15 m.

Carapace length (width for *M. spinosissimus*) frequency distributions were constructed for males and females and showed a unimodal distribution for all species over the range of individuals measured (Fig. 3). Carapace statistics (mean and standard error) for *P. argus*, *P. guttatus*, and *M. spinosissimus* were 52.0 mm ± 1.2 (±SE), 39.7 mm ± 1.6, and 91.7 mm ± 2.1 respectively. Significant correlations were made between CL and TL for both lobster spe-

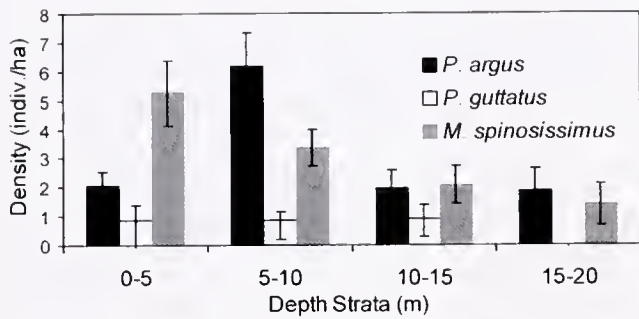


Figure 2. Density distributions across depth strata for *Panulirus argus*, *Panulirus guttatus*, and *Mithrax spinosissimus* populations in Bocas del Toro, Panama. Error bars represent standard error.

cies (*P. argus*, $y = 1.4178x - 5.1383$, $R^2 = 0.76$, $P < 0.05$; *P. guttatus*, $y = 1.6794x - 15.773$, $R^2 = 0.83$, $P < 0.05$) (Fig. 4). Male to female sex ratios for all species were: *P. argus*, 1:1.2; *P. guttatus*, 1.6:1; *M. spinosissimus*, 1.2:1. No *P. argus* females were

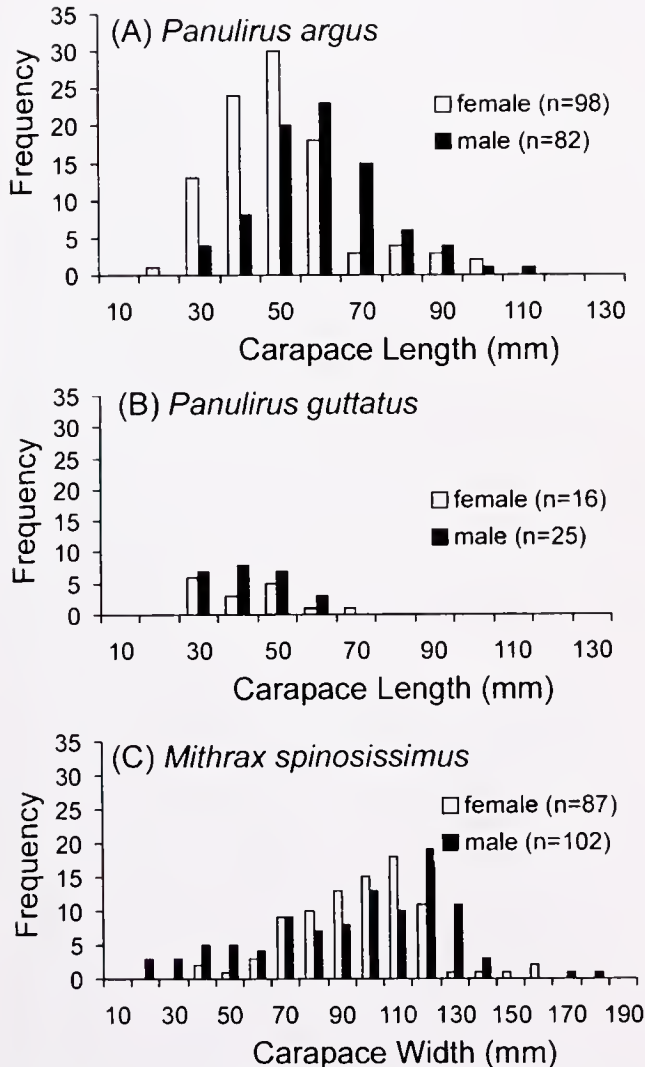


Figure 3. Frequency distributions of female and male (A) *Panulirus argus*, (B) *Panulirus guttatus*, and (C) *Mithrax spinosissimus* in Bocas del Toro, Panama.

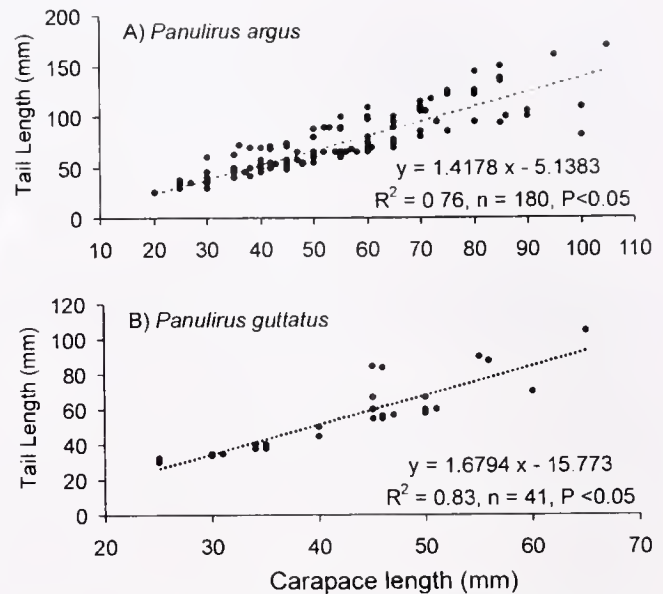


Figure 4. Tail length plotted against carapace length for female and male (A) *Panulirus argus* and (B) *Panulirus guttatus*, in Bocas del Toro, Panama.

found to be eggmass-bearing and only a single eggmass-bearing female *P. guttatus* (65-mm carapace) was encountered. The 50% maturity level for *M. spinosissimus* females, based on presence of eggmasses only, was found to be in the 81–90 mm carapace size class (Fig. 5).

DISCUSSION

The demand for spiny lobster resources throughout the Caribbean region over the last 50 years has resulted in depleted populations and an increase in the harvest of undersized and immature lobsters (King 1997, Cochrane & Chakalall 2001). This pattern may now be apparent in Panama with continual increases in landings (Fig. 6) and the documentation of removals of small lobsters in the San Blas archipelago (Castillo & Lessios 2001). This study may provide additional insights into depletions of *Panulirus argus* and *P. guttatus* populations in the Bocas del Toro through details on population abundance and distribution. This research also provides details on a poorly studied species, the West Indian spider crab (*Mithrax spinosissimus*), which may become a target of increased exploitation as a consequence of the depletion of other

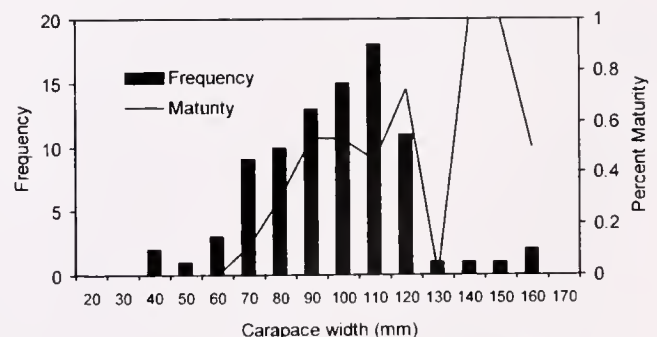


Figure 5. Frequency distributions and percent maturity of female *Mithrax spinosissimus* ($n = 87$) in Bocas del Toro, Panama.

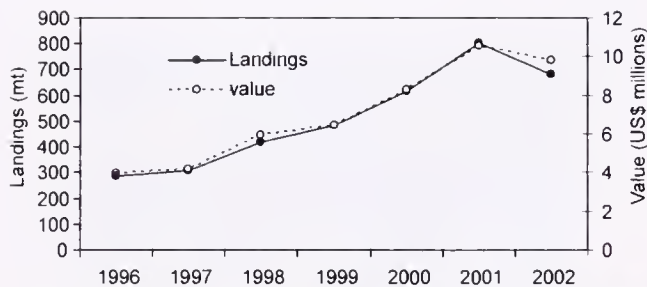


Figure 6. Total lobster landings (mt) and value (US\$ millions) of those landings in Panama (1996–2002). No detailed species composition is available (Cruz 2002).

more commercially preferred targets including lobster and conch (Tewfik & Guzman 2003) species.

The mean CL of both *P. argus* (52 mm) and *P. guttatus* (40 mm) are well below those reported in other areas of the Caribbean, including the San Blas archipelago (Yallondaro et al. 2001, Castillo & Lessios 2001, Losada-Tosteson et al. 2001). Even amongst fishery independent studies, the mean sizes in Bocas del Toro are small (Sharp et al. 1997, Tewfik et al. 1998). When examining size at sexual maturity and percent maturity of female lobsters, substantial depletions may be inferred when only a single egg-bearing *P. guttatus* (65 mm) was found in a total of 114 female lobsters examined of both species. Although our study was only conducted between April and September, it is generally considered that tropical species of *Panulirus* breed year around (Chubb 1994) with a significant proportion of egg-bearing females being identified over the period from February until November (Sharp et al. 1997, Castillo & Lessios 2001, Acosta & Robertson 2003). The small sizes and lack of mature individuals strongly suggest an over exploitation of the population as seen in other decapod fisheries (Abbe 2002). The importance of decreases in mean size and size at maturity is directly related to annual and lifetime egg production within a population (Pollock 1995) and ultimately levels of local recruitment. These links are known from other areas with simultaneous declines in spawning stock, recruitment, and individual size of adults (Lipcius & Stockhausen 2002). The skewed sex ratio towards males (1.6:1) in *P. guttatus* was as reported elsewhere (Sutcliffe 1953, Evans & Lockwood, 1994, Sharp et al. 1997, Losada-Tosteson et al. 2001). This skewness has been variably attributed to sex differences in survival, catchability, movement related to reproductive activity, and other behaviors (e.g., den guarding) and should be investigated further (Losada-Tosteson et al. 2001).

In contrast, morphological information obtained for *M. spinosissimus* including the large mean carapace width (92 mm) and high percentage of egg-bearing females, 45% with 50% maturity at approximately 85 mm, indicates that adults do exist. However, absolute comparisons of this *M. spinosissimus* population to others are difficult due to a shortage of other studies for this species in particular. Suggestions of a large literature base, and therefore understanding, on *Mithrax* spp. should be met with caution, as studies of the much larger *M. spinosissimus* are extremely limited.

Low overall density values for *P. argus* and *P. guttatus* compared with other fishery independent studies again suggest over-exploitation (Tewfik et al. 1998, Acosta & Robertson 2003). It is possible that *P. guttatus* was underestimated compared with *P. argus* due to its preference for dens that conceal the whole body and behaviors that include retreating back as far into the den as

possible to deter attacks from predators (Loranzo-Alvarez & Briones-Fourzan 2001). The absence of *P. guttatus* below 15 m conforms to the findings of other studies that this species is indeed a shallow water reef specialist (Sharp et al. 1997, Tewfik et al. 1998, Acosta & Robertson 2003). Absolute comparisons for *M. spinosissimus* are again impossible. The distribution of the three decapod species by depth (see Fig. 2) and area (see Fig. 1) are likely due to 2 main factors: resources and catchability. High densities may be facilitated by an abundance of shelter resources (complex reef habitats) or low catchability of targets due to low visibility for free-diving and/or rough surface conditions for the majority (87.5%) of fishers operating from small canoes. It is believed that a combination of both factors is responsible for the variability in density over the study area.

On the shelter resource, it is known that these three crustaceans do contend for occupancy of suitable den or crevice space. *P. argus* and *P. guttatus* may use the same dens, with *P. argus* on the floor and *P. guttatus* on the ceiling, but rarely simultaneously (Sharp et al. 1997). Observations of *P. argus* and *M. spinosissimus* indicate that the lobster can bar co-occupation by the crab but the crab cannot prevent the lobster from co-occupying a den (Hazlett & Rittschof 1975). The recruitment of juvenile *Panulirids* may also be limited by the availability of den or crevice space (Lipcius & Cobh 1994). Given the low densities of all three species and the observation that no more than one of the three species was seen occupying a single den it must be assumed that shelter resources are not limiting in Bocas del Toro at this time. However, the limitation of shelter resources among sympatric species in such systems may be of interest in the context of marine protected areas or fisheries reserves when ambient densities are increased through management.

In summary, it seems that the *Panulirid* populations of Bocas del Toro are in an overexploited state especially given the wide extent of the surveys throughout the archipelago. Recent interviews with the 192 lobster fishers within the area indicate that 55% are in favor of a ban on lobster with only 22% opposed. A further 18.5% would support a ban if other fishing possibilities existed (Cruz 2002). The strong support for management amongst resource users lends further credence to the view that lobsters are in short supply but also potentially bodes well for the rapid implementation of new protective legislation. Slight decreases in landings over the last 3 years within Bocas del Toro (18.63 mt in 1999, 17.95 mt in 2000, and 17.31 mt in 2001), in the context of national increases (see Fig. 6), may also reflect the difficulties that experienced fishers have locating lobsters.

A portion of Executive Decree No. 15 (March 30, 1981) describing size regulations for the harvest of lobster in Panama as a minimum CL of 60 mm and minimum TL of 120 mm seems inadequate and puzzling. Given the morphometric analyses conducted for *P. argus* in this study a 60-mm CL corresponds to an 80-mm TL. However, a 120-mm TL, by the same regression equation ($y = 1.4178x - 5.1383$), corresponds to an 88 mm CL. It seems obvious that a 60 mm minimum CL does not allow sufficient protection of juvenile *P. argus* because no mature individuals can be found at that size as well as the fact that the minimum CL and TL do not match. Furthermore, the average mean size of *P. argus* at maturity in the region ranges between 70–109 mm CL (Yallondaro et al. 2001). The existing minimum CL cannot be used for *P. guttatus* as virtually all individuals harvested would be illegal as is the case in Morrocoy National Park, Venezuela (Losada-Tosteson et al. 2001). It seems reasonable to raise the

minimum CL for *P. argus* to 88 mm to correspond to the existing and reasonable TL of 120 mm. This would allow many *P. argus* to become mature before being harvested and therefore increase the potential for contribution to future recruitment. Separate minimum sizes should be established for *P. guttatus* given their much smaller size. The suggestion that morphometric relationships established for either species in other parts of its range should be used for management is extremely dangerous and rejects the potential differences that may exist between populations. The exact minimum size for both lobster species should be based on more detailed local population CL when 50% of females are mature (egg-bearing) (Chubb 1994).

Further modifications to existing regulations could include a seasonal closure during reproductive peaks to allow undisturbed mating and spawning (Villegas et al. 1982). Alternatively, given the extremely low densities, a fishing ban could be implemented for several years to protect all lobsters allowing a rebuilding of the spawning stock, which presently seems to be low (Chiappone &

Sealy 2000, Goni et al. 2001). The exact nature of the management strategy for *M. spinosissimus* seems more difficult, given the extremely limited understanding we have of the species. The existence of mature crabs is a positive point. However, given the high pressure on many of the shallow-water marine resources of the archipelago further study is urgently required.

ACKNOWLEDGMENTS

The authors thank C. Guevara, A. Castillo, and W. Pomaire for providing invaluable assistance in the field and Z. Batista, I. Candanedo, M. Caswell, M. Endara, and G. Martinez for administrative support. J.M. Guevara developed the map in GIS. The Government of Panama provided all necessary permits to work in the country. This research was partially funded by The Nature Conservancy (35%) through the Theron Johnson and Katherine Ordway Stewardship Endowments and the Smithsonian Tropical Research Institute (65%).

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THE RELATIONSHIP BETWEEN SEAWEED DIET AND PURPLE INK PRODUCTION IN *APLYSIA DACTYLOMELA* RANG, 1828 (GASTROPODA: OPISTHOBRANCHIA) FROM NORTHEASTERN BRAZIL

LUIS ERNESTO ARRUDA BEZERRA,¹ ANA FONTENELE URANO CARVALHO,¹
LUCIANA AIRES BARREIRA,¹ VANESSA LUCIA RODRIGUES NOGUEIRA,¹
JOSÉ ROBERTO FEITOSA SILVA,¹ ILKA MARIA VASCONCELOS² AND
VÂNIA MARIA MACIEL MELO^{1,*}

¹Department of Biology Federal University of Ceará Campus Pici Fortaleza, Ceará - Brazil 60 455-760

²Department of Biochemistry and Molecular Biology Federal University of Ceará Campus Pici –
Fortaleza, Ceará Brazil

ABSTRACT *Aplysia dactylomela* is a large marine opisthobranch gastropod, which inhabits shallow tropical shoreline regions, eats red and green algae, and releases a purple ink when disturbed. Many functions have been proposed for this secretion and although there is no consensus on this, some of its constituents are believed to be derived from the red algal diet, it may provide the snail with a substantial survival advantage. *A. californica* only produces ink when it ingests red seaweeds. In some locations of the Northeastern Coast of Brazil, *A. dactylomela* is seen feeding only on green seaweeds, and yet it releases the ink. The aim of this work is to investigate this contradiction by studying the feeding habits of *A. dactylomela* and assessing the relationship between the algal diet and the purple ink. Feeding habits were investigated by field observation and by analysis of gut contents. Purple ink production was monitored by histologic analysis of the ink gland from sea hares kept in water tanks, fed with either red or green seaweeds. Composition and protein profile of the purple ink also were studied. Homologies between seaweed components and the purple ink were sought for by immunodiffusion techniques. Our findings are that the sea hare *A. dactylomela*, likewise other *Aplysia* species, needs to consume red seaweeds to be able to secrete the purple ink. The proteins of the ink seem to be synthesized by the sea hare itself and are not obtained directly from the diet, as is the case for the ink pigments.

KEY WORDS: *Aplysia dactylomela*, purple ink, ink gland, seaweed diet

INTRODUCTION

Aplysia dactylomela is a large marine opisthobranch gastropod which inhabits shallow tropical shoreline regions, eats red and green algae, and lays large numbers of fertilized eggs in string-like gelatinous masses close to the sea surface. It is hermaphroditic and nocturnally active, but may be exposed to sunlight as it rests during the day (Carefoot 1987). Sea hares probably are best known because of the purple ink they release when disturbed. This ink is secreted from the ink gland located on the edge of the mantle shelf, and the ability to produce purple ink is reported to be associated with a red seaweed-containing diet (Coelho et al. 1998). Of the 37 species of sea hare from the *Aplysia* genus, 30 can secrete purple ink (Nolen et al. 1995). Many functions have been proposed for this secretion, such as camouflage, alarm signal, pheromone, aposematism (use of color patterns by prey animals to signal their distastefulness to predators), bile excretion, predator deterrent, and cue of danger (Johnson & Willows 1999). Probably the ink has more than one role in the biology of sea hares. Although there is no consensus on the biologic function of the ink, it is known that some of its constituents are derived from red algae in the diet (Prince et al. 1998), and it provides the snail with a substantial survival advantage. Recent work by Prince et al. (1998) on the ink glands of *A. californica* has improved our understanding of ink gland structure and processing and secretion of purple ink.

In addition to studies of the ecologic and biologic aspects of chemical defense in sea hares, special attention also has been paid to the isolation and characterization of new bioactive substances from the purple ink. Thus, many bioactive substances have been isolated from the purple ink, including proteins with antibacterial

activity in *A. punctata* (Nistratova et al. 1992), antitumor and cytolytic activities in *Dolabella auricularia*, (Kisugi et al. 1989, Yamazaki et al. 1989a) and cytolytic and antibacterial activities in *A. kurodai* (Yamazaki et al. 1989b, Yamazaki et al. 1990). In Brazil, antibacterial and hemagglutinating activities were described for the purple ink of *A. dactylomela* (Melo et al. 1998; Melo et al. 2000).

On the northeastern Coast of Brazil, there is an abundance of seaweed species that support the great biodiversity of this tropical region. On some beaches there is a predominance of green seaweeds (mainly *Ulva fasciata*), whilst on others, red seaweeds prevail. On the beaches dominated by green seaweed, *A. dactylomela* is seen feeding mainly on them and yet releases the purple ink when disturbed. This fact could be intriguing, considering that other *Aplysia* species need to consume red seaweeds to be able to produce the ink. To solve this apparent contradiction this study investigates the feeding habits of *A. dactylomela* and assesses the relationship between the algal diet and the purple ink.

MATERIALS AND METHODS

Determination of Feeding Habits

Feeding habits of *A. dactylomela* were observed in 2 beaches of Ceará State, Northeast of Brazil, between August 2000 and July 2001. One beach was particularly rich in the green seaweed *Ulva fasciata* (beach 1, 38°38'48"W and 3°41'24"S); whereas, the other was densely rich in the red species, particularly *Hypnea musciformis* and *Gracilaria* spp. (beach 2, 39°25'45"W and 3°22'18"S). Feeding habits of sea hare were examined monthly during daytime at low tide by observing the seaweed species consumed in the field

*Corresponding author. E-mail: vmmelo@ufc.br

and by dissecting under a stereomicroscope the gut contents of the sea hares and capturing the images with a digital camera (Sony-Mavica, Japan).

Histologic Analysis of the Ink Gland

Specimens of *A. dactylomela* were collected during low tide and transported to the laboratory in a container with seawater. Some specimens were de-inked by squeezing them gently for a few minutes outside the water and then kept in seawater tanks for 15 days with unialgal diets (*U. fasciata* or *Gracilaria* sp.) to observe whether there was purple ink production. At the end of this period, sea hares were anaesthetized by storage in a refrigerator and then dissected carefully to remove the ink gland.

For light microscopy, ink gland samples were fixed with Bouin mixture (saturated picric acid solution, 40% formaldehyde, glacial acetic acid (15: 5:1 v/v) and embedded in paraffin. Tissue sections (5 μ m) were stained with hematoxylin and eosin (Junqueira & Junqueira, 1983) and with bromophenol blue for detection of proteins (Pearse 1960). Histologic sections were observed under a microscope, and photographs were taken.

Chemical Composition of Purple Ink

Purple ink was analyzed for contents of water, protein (total nitrogen), reduced carbohydrate, lipid, and ash. For the water determination, 1g of ink was dehydrated in an oven at 100°C to 110°C to constant weight. Total nitrogen was determined in samples of freeze-dried purple ink by micro-Kjeldahl digestion (Baethgen & Alley 1989). The content of reduced carbohydrate was determined according to Dubois et al. (1956). Lipid content was determined by n-hexane Soxhlet extraction, and ash was quantified by heating 1g of ink in a muffle furnace at 620°C for 18 h.

Amino Acid Analysis

Dry purple ink (1 mg) was hydrolyzed in 1 mL 6 M HCl with 1% phenol (w/v), in a sealed glass tube under N₂, at 110°C for 22 h. After hydrolysis, HCl and phenol were removed by evaporation, and the residue was analyzed in a Biochrom 20 (Pharmacia-LKB) amino acid analyzer.

Electrophoretic Profile of Ink Proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the presence of beta-mercaptoethanol according to the method of Laemmli (1970). Proteins were stained with silver nitrate (Blum et al. 1987). Bovine serum albumin (66.0 kDa), egg albumin (45.0 kDa), porcine pepsin (34.7 kDa), bovine β -lactoglobulin (18.4 kDa), and egg lysozyme (14.3 kDa) were used as standards (Sigma Co., USA).

Seaweed Protein Extraction and Determination

Proteins were extracted from fresh samples of the green seaweed *U. fasciata* and the red species *H. musciformis* and *Gracilaria* sp. The samples were ground with a mortar and pestle with the following buffers at 50 mM: Glycine-HCl, pH 2.6; Tris-HCl, pH 7.0; and sodium borate, pH 9.0. The extracts of *U. fasciata* and *H. musciformis* were prepared at the proportion of 1:3 (m/v) and that of *Gracilaria* sp. at 1:5 (m/v). The extracts were filtered through a nylon tissue and centrifuged at 15,000 g for 10 min at 4°C. The supernatants (crude extracts) had the protein content determined according to Bradford (1976) with bovine serum albumin (BSA) as the standard (purchased from Sigma Co., USA).

Anti-Purple Ink Polyclonal Antibody

Anti-purple ink polyclonal antibody was developed in a 3-month-old albino rabbit, which was immunized by intramuscular injection with 1 mg of purple ink, previously dialyzed and freeze-dried and then dissolved in 1 mL of sterile saline solution containing incomplete Freund adjuvant, 1:1 v/v (Sigma Co.). Booster injections were given without adjuvant subcutaneously on the 14th, 21st, 28th, and 35th days after the primary injection. Immune serum was obtained by blood sampling from the marginal ear vein on the 21st, 28th, and 35th days.

Immunodiffusion Assay

Immunologic relationships between protein extracts from seaweeds and the purple ink were assessed by the Ouchterlony double diffusion method (Hudson & Hay 1989). The assays were done in 1% agar plates prepared with 20 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl and 0.02% sodium azide. After solidification of the agar, wells were made with a Pasteur pipette, and 30 μ L of each seaweed extract was applied into each well and tested against the anti-purple ink antibodies applied in the central well. After incubation at room temperature for 48 hours, the gels were washed with 150 mM NaCl, dried, and stained with Coomassie brilliant blue for visualization of the precipitation arcs.

RESULTS

Feeding Habits

Sea hares from beach 1 were frequently surrounded by the green alga *U. fasciata*, and to a much less extent by red alga species (*Gracilaria* spp. and *H. musciformis*). In beach 2, however, the snails were surrounded by red algae, mainly *H. musciformis*, and some brown species. The analysis of gut contents from specimens collected showed that the snails ate mainly those alga species surrounding them. Those from beach 1 had gut contents comprised of approximately 70% *U. fasciata* (and traces of red species); whereas, those from beach 2 contained over 90% *H. musciformis* (photography not included).

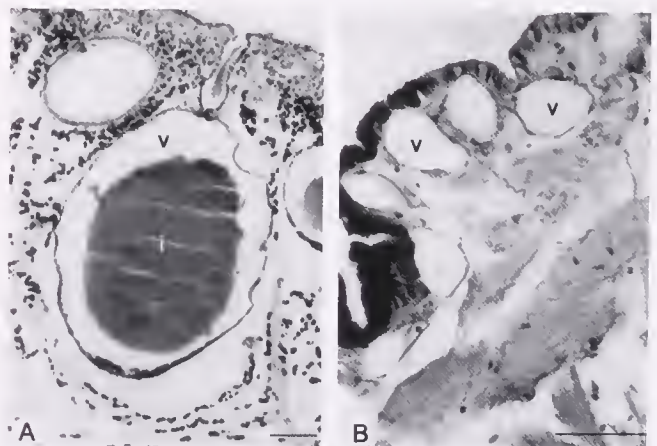


Figure 1. Light micrographs of ink glands in red-seaweed-fed (A) and green-seaweed-fed *Aplysia dactylomela* (B). Vesicles (v) were full of ink (i) in red-seaweed-fed sea hares and empty in green-seaweed-fed. Scale bars: A, B, 100 μ m.

TABLE 1.

Chemical analysis of the purple ink from *Aplysia dactylomela*.

Components	% Dry Basis
Total protein (N \times 6.25)	64.87 \pm 2.56
Reduced carbohydrate	9.07 \pm 1.84
Lipid	2.20 \pm 0.40
Ash	2.72 \pm 0.20

Values are means \pm standard deviation of at least triplicate analyses.*Histologic Analysis of the Ink Gland*

Histologic analysis showed that ink gland tissues from animals whose gut contained mainly green algae had most vesicles empty. The same was observed with the animals kept on the *U. fasciata* diet in the laboratory tank. By contrast, the ink glands of animals from beach 2, which consumed mainly red alga species, had most vesicles full (Fig. 1).

Chemical Composition of Purple Ink

The purple ink is composed of approximately 99.5% water. Purple ink proximal chemical analysis, on dry basis, is described in Table 1, being comprised mainly of proteins (over 60%). Regardless of diet, the ink had the same basic composition of protein, carbohydrate, lipid, and ash.

Amino Acid Composition

The results of amino acid composition (Table 2) showed that the purple ink contains high levels of acidic amino acids, glutamic, and aspartic acid and very low content of sulfur amino acids.

Electrophoretic Profile of Ink Proteins

The purple ink of sea hares from beaches 1 and 2 showed similar electrophoretic profiles in terms of mobility and intensity

TABLE 2.

Amino acid composition of purple ink of *Aplysia dactylomela*

Amino Acid Residue	g of Amino Acid in 100 g of Dry Matter
Ala	6.03
Arg	5.07
Asx	11.77
Cys	1.43
Glx	11.95
Gly	6.48
His	1.12
Ile	3.68
Leu	7.10
Lys	4.70
Met	1.55
Phe	4.55
Pro	8.98
Ser	5.78
Thr	7.54
Trp	ND
Tyr	5.46
Val	7.59

ND = not determined.

of protein bands (Fig. 2). Two prominent bands were observed at 60 and 45 kDa, several minor bands span the apparent molecular mass range 20–30 kDa, whilst others were approximately 15 and few below 14 kDa.

Serological Relationship Between the Purple Ink and Seaweed Extracts

The immunodiffusion assay showed that the anti-purple ink antibodies reacted with the ink but did not recognize any seaweed component extracted either with acid, neutral or alkaline buffer (photography not included). This indicates, therefore, that no immunologic reactivity exists between components of algal diet and the purple ink.

DISCUSSION

This study describes the relationship between the seaweeds consumed by the sea hare *A. dactylomela* and its purple ink.

Although it has been reported that the secretion of purple ink by sea hares is associated with their consumption of red seaweeds (Prince et al. 1998), specimens that consumed mainly green seaweeds (beach 1) also were seen to release the purple ink. This contradiction was solved when the analysis of gut contents showed the presence of traces of red seaweeds, indicating that minor consumption of red species was enough to support the ink production.

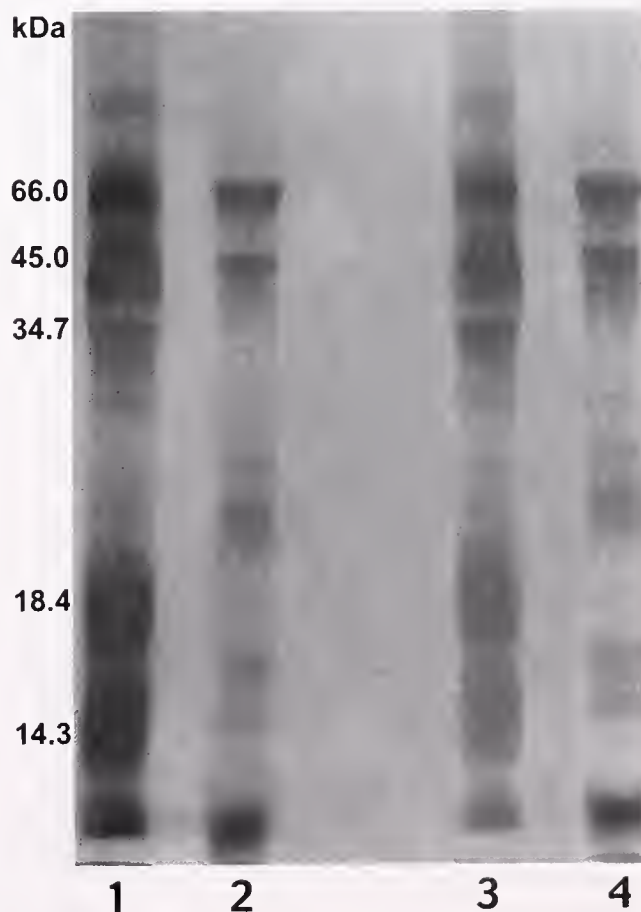


Figure 2. SDS-Polyacrylamide gel electrophoresis. Lanes 1 and 3, molecular mass markers; lane 2 purple ink from *Aplysia dactylomela* collected at beach 1 (green seaweed rich) and lane 4 purple ink from specimens from beach 2 (red seaweed rich).

To prove that *A. dactylomela*, like *A. californica* (Prince et al. 1998; Coelho et al. 1998), only produces ink when it ingests red seaweeds; laboratory experiments were conducted with sea hares receiving the green seaweed *U. fasciata*, or the red *Gracilaria* sp., as the sole source of food. Our light microscopy studies of the ink gland of sea hares consuming only *U. fasciata* showed that most of the vesicles were devoid of purple ink; whereas, individuals that consumed red seaweeds had full vesicles in the ink gland. These observations confirm the necessity of red-seaweed consumption for *A. dactylomela*, and probably other sea hares of the same genus, to be able to secrete the purple ink.

The chemical analysis of the purple ink showed that, regardless of the main seaweed species consumed by the sea hare, the content of protein in the ink was the same. This could indicate that either diet, rich in green or red seaweeds, must be able to supply the amino acids necessary for ink protein synthesis. Our analysis of amino acid composition showed that the purple ink is rich in acidic amino acids and has very low content of sulfur amino acids, reflecting the amino acid composition of both seaweeds (Ramos et al. 2000). Likewise, the electrophoretic profile of the ink proteins was always the same, regardless of the sample origin (beach 1 or 2), time of the year, or sea hare age. The finding that the anti-ink antiserum did not recognize any protein of the seaweed extracts suggests that the sea hare itself synthesizes the ink proteins and that they are not obtained directly from the diet, as is the case for

the ink pigments (Troxler et al. 1981; MacColl et al. 1990). Coelho et al. (1998) previously suggested that the pigment of *A. californica* purple ink is of algal origin, but the proteins are not. Thus, there seems to be no doubt about the origin of the ink substances (pigments and proteins) in *A. dactylomela*. Nevertheless, the roles in the purple ink have not yet been completely elucidated. Several biologic activities have been described *in vitro* for isolated proteins (Kisugi et al. 1989; Yamazaki et al. 1989a; Yamazaki et al. 1989b; Yamazaki et al. 1990; Nistratova et al. 1992; Melo et al. 1998; Melo et al. 2000) and pigments (Wessels et al. 2000) from sea hare ink, and all give support to a defense role.

CONCLUSIONS

The sea hare *A. dactylomela*, and possibly other *Aplysia* species, needs to consume red seaweeds to be able to secrete the purple ink. The pigment is from algal origin; whereas, the proteins are not. The protein content and composition seem to be always the same, regardless of the seaweed species consumed.

ACKNOWLEDGMENTS

The authors thank PET/CAPES for a BS fellowship to L.E.A. Bezerra, CNPq for grants received and to the students R.C.N. Amorim, J.E.A. Beserra-Jr, and M. Thadeo for their assistance during the field work.

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NUCLEIC ACID DERIVED INDICES OR INSTANTANEOUS GROWTH RATE AS TOOLS TO DETERMINE DIFFERENT NUTRITIONAL CONDITION IN CUTTLEFISH (*SEPIA OFFICINALIS*, LINNAEUS 1758) HATCHLINGS

ANTÓNIO V. SYKES,* PEDRO M. DOMINGUES AND JOSÉ P. ANDRADE

C.C. MAR Universidade do Algarve F.C.M.A., Campus de Gambelas 8000, 817 Faro, Portugal

ABSTRACT Two groups of 100 cuttlefish hatchlings each were used to determine the duration of the yolk reserves, during which growth can be obtained with no food supply. One group was fed live grass shrimp *ad libitum* from the 3rd day of life onwards, while the other was starved during the whole experiment. The experiment lasted 7 days, because this was the 1st day where a negative growth rate was obtained. Nucleic acid derived indices and instantaneous growth rates (IGR) were used as a way to describe their condition, when all the yolk reserves would be exhausted and to determine the most accurate tool to express growth and condition. RNA/DNA ratios described clearly the differences ($P < 0.05$) between fed and starved hatchlings cultured at $23 \pm 2^\circ\text{C}$, especially from day 4 onwards. Correlation between IGR and RNA/DNA ratios was only attained for the starved population ($R = 0.90$; $P = 0.0374$). DNA concentration was identical ($P > 0.05$) between groups and did not display a clear pattern with increasing age. Correlation between IGR and DNA was only attained for the fed population ($R = -0.90$; $P = 0.0374$). RNA concentration values were different from day 5 onwards. Any of the nucleic acid derived indexes were not accurate enough to express growth and condition, so IGR seems to be the most accurate and inexpensive way to describe hatchlings growth and condition in controlled conditions.

KEY WORDS: condition indices, cuttlefish, DNA/RNA ratios, growth rates, hatchlings, accuracy methodologies, *Sepia*

INTRODUCTION

When culturing any species in intensive aquaculture, time of first feeding is of extreme importance. Food should be available when larvae or hatchlings are finishing the absorption of the inner yolk reserves and starting to feed externally. Cuttlefish are known to be voracious feeders throughout their life cycle and accept a wide range of prey (Guerra 1985, Castro & Guerra 1989, Pinzon du Sel & Daguzan 1992, Domingues et al. 2001a, Domingues et al. 2003). Some authors (Wells 1958, Richard 1971, Richard 1975, Boletzky 1983, Boletzky 1987, and Nixon 1985) have studied the embryonic development and the early life history of cuttlefish and set the end of the inner yolk reserves around the 3rd day of life. However, according to Domingues et al. (2001b), cuttlefish in unfed or poor nutritional condition (as result of prey with poor nutritional profile) can last as much as 20 days. The only way to determine optimal timetables for first feeding, thus optimizing cuttlefish production, is the use of condition analysis. Condition is a measure of the physical status or well being of an animal and may be used to evaluate growth or survival rates (Bolger & Conolly 1989, Ferron & Leggett 1994).

Nutritional condition of larval stages plays an important role in the knowledge of the recruitment of marine species. Nutritional condition of fish larvae can be evaluated using several methods: morphometric, histologic, and biochemical (Chícharo 1993). However, costs and results associated with each methodology are not similar between them. So, the main questions are which methodology to choose when trying to evaluate the condition of the animal, and the compromise between costs and accuracy of results to achieve. The use of morphometric methodologies are standards in most aquaculture and fishery laboratories around the world. Nowadays, biochemical methodologies related to nucleic acid derived indices are becoming standards also.

One of the most commonly used methods for determining the nutritional condition and growth is the RNA/DNA ratio (Buckley 1979, Buckley 1980, Buckley & Lough 1987, Clemmesen 1988,

Clemmesen 1990, Robinson & Ware 1988). The use of this methodology is based on the assumption that DNA is present in constant concentrations, under changing environmental conditions or during starvation (Richard et al. 1991), whereas RNA varies, decreasing in starving animals (Buckley 1984, Buckley & Lough 1987). According to Bulow (1987), RNA concentrations are higher in tissues with faster growth rates or with a higher rate of protein synthesis. The RNA/DNA ratio indicates quantities/concentrations of RNA per cell and is the most accurate when estimating tecidular proteosynthetic activity (Bulow 1987, Buckley 1981, Buckley 1984). The use of RNA concentration in a tissue as an indicator of growth is based on the assumption that RNA is related to the potential for protein synthesis (Houlihan 1991). However, large variation in the RNA/DNA ratio may occur in fed larvae (Clemmesen 1988, Raae et al. 1988). Recent studies suggest that the RNA/DNA ratio is one of the best indicators of the nutritional condition of several marine organisms (Clemmesen 1994, Bailey et al. 1995, Chícharo 1997, Chícharo et al. 1998, Chícharo et al. 2001).

Biochemical methodologies have been proposed to evaluate condition in post-hatch and juvenile cuttlefish (Clarke et al. 1989, Pierce et al. 1999, Koueta et al. 2000). One of those is the RNA/DNA nutritional condition ratio. However, until now, only RNA concentrations in the muscle were shown to be directly correlated with growth in *Octopus vulgaris* (Houlihan et al. 1990) and *Sepia officinalis* (Castro & Lee 1994).

The objective of this research is to determine: (1) how long cuttlefish hatchlings could survive on inner yolk reserves after hatching, using nucleic acid derived indices (RNA/DNA ratio, [DNA]/g and [RNA]/g) and instantaneous growth rates (IGR) as ways to describe their growth and condition and (2) the best descriptor for condition, based on a compromise of money spent, results achieved, and when to apply it.

MATERIAL & METHODS

Experimental Conditions

A group of 100 cuttlefish hatchlings was used to determine the maximum starvation period, while another group of 100 hatchlings

*Corresponding author. E-mail: asykes@ualg.pt

(control) were fed live grass shrimp (*Palaemonetes varians*) captured from nature. The control group was fed ad libitum from day 3 to 7. Both groups were placed in baskets (5.4 L water volume, 1-mm mesh) which were in 250 L tanks of a flow-through system with a UV unit, described in Domingues et al. (2001b and 2002). Water flow was of 12 L/h. Water temperature was of $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and salinity was of 37 ± 2 PSU; lights were running on a 12 h per day basis. This system ensured an excellent water quality, necessary for hatchling culture.

Because several authors reported that cuttlefish hatchlings start external feeding on the 3rd day of life (Richard 1971, Richard 1975, Boletzky 1983), this was the starting period for collection of data on growth, survival, and condition. However, two samples of 20 newly born hatchlings were taken to determine RNA and DNA concentrations and ratio at time of hatching. The experiment lasted until the 7th day, because, at this time, negative growth rates for the starved animals started to be obtained. Each day 20 hatchlings were collected from each group, weighed, frozen, and stored (at -80°C) for later nutritional condition analysis. Collected data were used to calculate: (1) mean weight; (2) mean instantaneous growth rate (IGR) ($\%\text{BW}\cdot\text{d}^{-1}$) = $(\ln W_2 - \ln W_1)/t \times 100$, where W_2 and W_1 are the final and initial weight respectively, \ln the natural logarithm and t the number of days of the time period; and (3) survival rates.

Nucleic Acid Determinations

Methods for the determination of nutritional condition were adapted from Esteves et al. (2000) and Chicharro et al. (2001).

Purification of tissues was achieved by homogenizing each cuttlefish in 2.5 ml TRIS-HCl solution for 5 sec with an ultraturrax unit (IKA Labortechnik), and 10 sec in an ultrasonic homogenizer unit (4710 Series, Cole Parmer Instruments Co.). This methodology was applied to obtain maximum homogeneity of tissues and disruption of cells, as well as a total destruction of the cuttlebone. After that, the homogenate was washed with 50 μl 3% sarcosine Tris-HCl and 1.35 mL Tris-HCl (Trizma, pH = 8) solutions and centrifuged during 5 min, at 9860 RPM and 4°C . Subsequent fluorescence-photometric measurements were done. DNA+RNA measurements were done combining 0.2 mL of supernatant with 0.4 mL of Tris-NaCl (Trizma, pH = 7.5) and 0.05 mL ethidium bromide (EB) (0.1 mg/mL), which is a nucleic fluorochrome dye specifically designed. DNA measurements were done combining 0.2 mL of supernatant with 0.35 mL of Tris-NaCl (Trizma, pH = 7.5) and 0.05 mL of ribonuclease A (Type-II A, Sigma, 0.12 $\mu\text{g}\cdot\text{mL}^{-1}$). After that, this mixture was incubated in a water bath for 30 min at 37°C and allowed to reach room temperature for 20 min. Same quantities of ethidium bromide (EB) (0.1 mg/mL) were then used to stain the DNA and DNA+RNA in mixture samples. Each cuttlefish sample was analyzed in triplicate. Values of DNA and RNA were then estimated calculating the mean value of the 3 replicates.

Determinations of nucleic acid concentration were identical for both DNA+RNA and DNA measurements. The fluorescence due to total RNA was then calculated using the difference between total fluorescence (RNA+DNA) and the fluorescence after the application of ribonuclease A. This second fluorescence is assumed to be due to DNA, after the subtraction of the self-fluorescence by the enzyme. Both fluorescences were determined by exciting at 365 nm and reading at 590 nm with a Hitachi spectrofluorometer (model 650-10). Concentrations of both DNA and RNA were de-

termined using standard curves of known concentrations of calf DNA and yeast RNA, after carrying out the same methodology as for the samples.

Data Analysis

Mann-Whitney tests (Zar 1984) were used to determine differences in IGR, RNA/DNA, [DNA]/g larvae and [RNA]/g larvae between fed and unfed hatchlings. Spearman rank order correlation tests (Zar 1984) were used to determine correlations between IGRs and RNA/DNA and IGR and DNA of fed and unfed hatchlings.

RESULTS

IGR and Survival

Both starved and fed cuttlefish hatchlings showed different IGRs ($P < 0.05$) (Fig. 1). Starved cuttlefish group showed a steady decline in IGR from values of about $6\% \text{ BW}\cdot\text{d}^{-1}$ (day 4) to negative values of approximately $-1.4\% \text{ BW}\cdot\text{d}^{-1}$ (day 7). Fed hatchlings showed similar values of IGR (minimum of 3.6% and maximum of $8.4\% \text{ BW}\cdot\text{d}^{-1}$) to the ones obtained by Sykes (2003).

Mortality was low and only 6 out of 100 hatchlings (6%) died in each group being tested.

Nucleic Acid Derived Indices

Standard calibration curves were obtained for both DNA and RNA. DNA calibration can be described using linear regression analysis by the equation $y = 13.961 \times + 3.7498$ ($r^2 = 0.996$; $n = 588$). RNA calibration can be described using linear regression analysis by the equation $y = 4.3829 \times + 3.9095$ ($r^2 = 0.992$; $n = 315$).

Hatchling condition was significantly different between fed and unfed hatchlings ($P < 0.05$). RNA/DNA ratio of fed hatchlings increased, whereas in starved hatchlings decreased, throughout the experiment (Fig. 2). Maximum values of RNA/DNA ratio (1.60 at day 7) were obtained in fed hatchlings whereas minimum values (0.00 at day 7) were obtained in starved hatchlings. Standard deviation of RNA/DNA ratio was high for both groups.

DNA concentration per g of body weight showed no significant differences ($P > 0.05$) between concentrations until day 4, whereas during day 5 to day 7, DNA concentrations were significantly different ($P < 0.05$) between both groups (Fig. 3). DNA concentration was higher in starved than in fed hatchlings. Maximum values were obtained in starved hatchlings ($13.04 \mu\text{g/g}$ at day 5) whereas minimum values were obtained in fed hatchlings (5.23



Figure 1. Growth rates of starved and fed hatchlings.

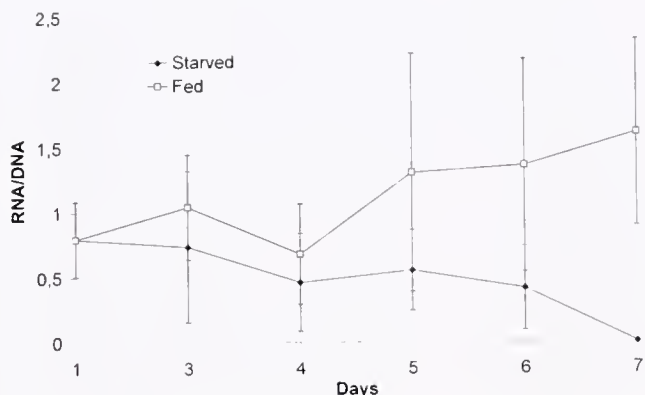


Figure 2. RNA/DNA ratios of starved and fed hatchlings. Vertical lines represent standard deviations.

$\mu\text{g/g}$ at day 7). Standard deviation was higher in starved than in fed hatchlings.

RNA concentration per g of body weight showed no significant differences ($P > 0.05$) in concentrations only for day 4 and 5 (Fig. 4). RNA concentration was higher in fed than in starved hatchlings. Maximum values were obtained in fed hatchlings ($9.05 \mu\text{g/g}$ at day 5) whereas minimum values were obtained in starved hatchlings ($0.00 \mu\text{g/g}$ at day 7). Standard deviation was high in both groups.

Spearman rank order correlation showed correlation between IGR and RNA/DNA for the starved group ($R = 0.90$; $P = 0.0374$), but it did not show correlation for the group fed with grass shrimp ($R = 0.80$; $P = 0.1041$) (Fig. 5 and Fig. 6). IGR and RNA/DNA decreased with increasing age for the starved group while the opposite occurred for the fed group.

Correlation of IGR and DNA for both fed and unfed hatchlings using Spearman rank order correlation showed inconclusive results. Results of the correlation between IGR and DNA for the fed group were significant ($R = -0.90$; $P = 0.0374$) whereas the correlation for IGR and DNA of the unfed group were not significant ($R = -0.30$; $P = 0.6238$).

DISCUSSION

According to Berrigan and Charnov (1994), both low temperature and below normal feeding rates will reduce growth rates in most ectotherms. Forsythe et al. (1991) stated that temperature and

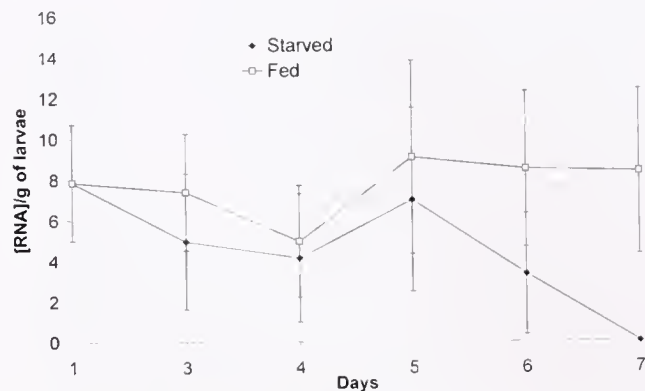


Figure 4. [RNA]/g of larvae of starved and fed hatchlings. Vertical lines represent standard deviations.

ration levels are the main factors determining variations in cephalopod growth rates. Because temperature was the same for both groups, differences in growth were directly related to feeding or starving.

Fed cuttlefish hatchlings readily accepted grass shrimp from the 1st day of feeding (day 3) onwards. Results obtained lead us to believe that inner yolk reserves should last at least to the 4th day after hatching, at a temperature of $23^\circ\text{C} \pm 2^\circ\text{C}$. This assumption is made based upon the decline in both patterns of IGR and RNA/DNA ratios for fed hatchlings on the 4th day, and the fast decline in starved hatchlings from the 5th day onwards. However, time for the first feeding should be established for the 3rd day after hatching because of cuttlefish energy consumption when capturing grass shrimp. If hatchlings are first fed later than this 3rd day, it will probably originate higher mortality values at this optimal culture temperature, because their capability to capture grass shrimp will be greatly diminished by lack of energy resources. This lack of energy resources is supposed to be related to the ending of the inner yolk reserves and their metabolization. The establishment of a time for the first feeding is particularly important because cuttlefish eggs do not usually hatch in the same day as fish do, but instead, they take their time hatching. This problem is caused by females intermittent spawning (Boletzky 1987b).

Previous studies showed that growth rates have been the most accurate way of measuring cuttlefish growth and condition for the first weeks of life. Instantaneous growth rates have been calculated for a number of cephalopods (Forsythe 1993, Lee 1994, Sykes 2003) and can give some indication about cuttlefish condition over time. Unfed or underfed animals will display negative or very slight variations in IGR during survey time, respectively. On the other hand, well-fed animals will display positive IGR. In the present experiment, fed and starved hatchlings showed these patterns.

Clarke et al. (1989) reported that in juvenile cuttlefish the RNA/DNA ratio was positively correlated with growth in individuals kept at 17.5°C , but no correlation was found on those kept at 12°C . According to the same author, RNA/DNA ratios of cuttlefish with low growth rates were similar to those reported for fish larvae; nucleic acid ratios at higher growth rates were higher than those reported so far. These authors also stated that higher values of RNA/DNA ratio could be related to the very high growth rates of cephalopods. In the present study, RNA/DNA ratios clearly described the differences between fed and starved hatchlings cul-

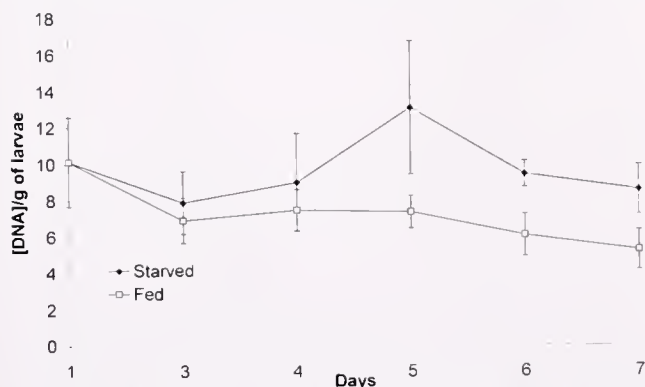


Figure 3. [DNA]/g of larvae of starved and fed hatchlings. Vertical lines represent standard deviations.

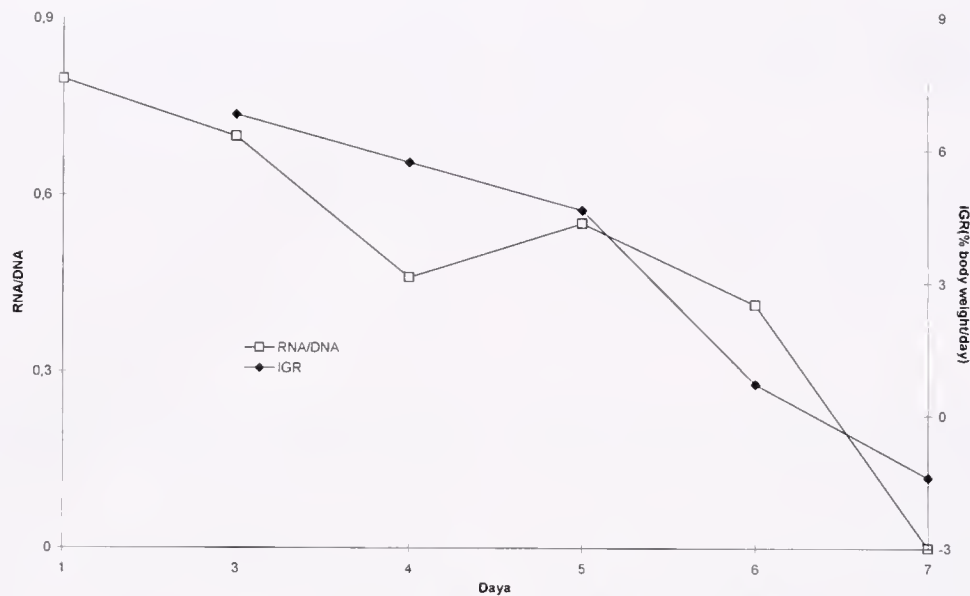


Figure 5. RNA/DNA ratios and IGR for starved hatchlings.

tured at $23 \pm 2^\circ\text{C}$, especially from day 4 onwards. However, RNA/DNA ratio results obtained in the present work were similar to those reported for fish larvae, and not higher as expected. This is particularly important because at this temperature cuttlefish will grow exponentially, as reported by Lee (1994) and more recently by Domingues et al. (2001a, 2002) and Sykes (2003). It seems that although feeding and growth rates are extremely high at this time of their life cycle, a correlation between growth and condition cannot be achieved. In fact, for the present study, when trying to establish a correlation between IGR and RNA/DNA ratios for both fed and starved groups, results were not conclusive. Although the present results showed a similar pattern, a statistical correlation was only observed for the starved group. According to Clarke

et al. (1989), if the RNA/DNA ratio is to be used as a tool in ecologic studies, there is a need for this relationship of RNA/DNA ratio with the nutritional status and growth rates to be precise. The same authors also add that RNA/DNA ratios would only allow distinction between zero, average and maximal growth. Castro & Lee (1994) stated that RNA/DNA ratio might not always be the best growth indicator for *S. officinalis*. So, RNA/DNA ratios are not accurate enough for determination of cuttlefish condition, but they will allow distinction between starved and fed animals.

These results indicate that DNA concentration should be of great importance when describing nutritional condition of cuttlefish hatchlings rather than RNA/DNA ratios. DNA concentration

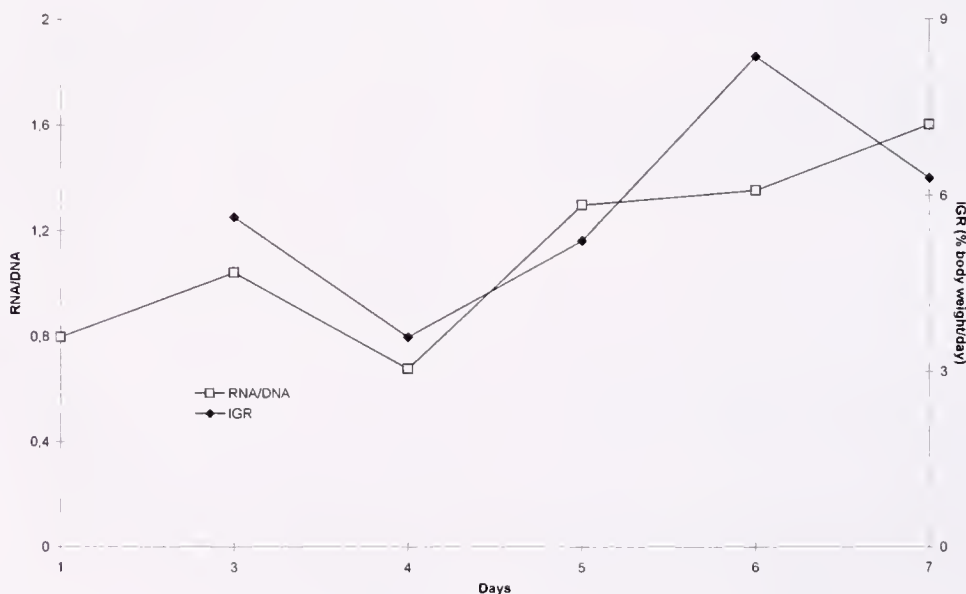


Figure 6. RNA/DNA ratios and IGR for fed hatchlings.

seems to be inversely correlated with IGR. Higher values of DNA were obtained when IGR displayed the lower values. Clarke et al. (1989) reported that the percentage of DNA/dry weight was much more stable in fed larvae than the RNA/DNA ratio. Chicharo (1993) stated that DNA concentration of fish larvae was especially sensitive to starving conditions after complete depletion of yolk reserves. It seems that the same happens with cuttlefish hatchlings. Bergeron and Person Le-Ruyet (1997) suggested that DNA concentration per g of body weight would be a better and simpler index of larval nutritional status than the RNA/DNA ratios, because it was more stable in fed hatchlings, and the methodology used to measure DNA alone would be easier, more sensitive, and less expensive than RNA/DNA ratios. However, correlation between IGR and DNA was only statistical significant for the fed group, and so DNA concentration is also not accurate enough to describe growth in cuttlefish.

Values obtained for RNA concentration in this study should describe starved hatchling condition and minimum or non-existent rate of protein synthesis, at $23\text{ C} \pm 2\text{ C}$. Mathers et al. (1994) found that an increase in protein content in fish larvae was not associated with similar increase in RNA concentration. According to Pierce et al. (1999) there is no direct relation between RNA concentration and growth rates in cephalopod species like *Loligo forbesi*, *Eledone cirrhosa* and *Octopus vulgaris*. The work of Castro and Lee (1994) showed, however, that the RNA contents in mantle muscle could be used as short-term indicator of instantaneous growth rate and condition of cuttlefish. More recently, Koueta et al. (2000) found that changes in RNA content of muscle were related to growth in *S. officinalis* hatchlings. The same author also stated that RNA content of the muscle increased significantly between underfed (low ration) and maintenance fed (medium ration) groups and were according to Bulow's (1987) findings for fish. Results from this study fall within the condition results obtained by Castro and Lee (1994) and Koueta et al. (2000), with a clear distinction between fed and starved animals. However, they are once again not accurate enough to describe growth because, in the present study, RNA concentration had great variations between animals that were under the same feeding conditions.

According to Richard et al. (1991), all methods for condition analysis have their limits, some are too time-consuming while others require large samples or are valid only for particular larval stages. Last but not the least, the amount of money spent on this kind of methodology is extremely high and results are comparatively poor in accuracy. This study shows that time tables for the first feeding time can be obtained easily without using nucleic acid

derived indexes by IGR. In spite of the lack of correlation between IGR and RNA/DNA ratios in fed hatchlings, IGR still seems to be the most appropriate evaluator of cuttlefish growth and condition in controlled environments because it can be applied throughout the life cycle and is less expensive and time consuming. Forsythe (1993) stated that exponential growth patterns of some temperate cephalopods mean that very small changes in the growth rates of juveniles will translate into large differences in the adults. These small changes cannot be measured by nucleic acid derived indexes because of the variation associated with this method and described earlier. Nevertheless, it is possible that nucleic acid derived indexes could be used as tools to evaluate recruitment and wild larval condition and to evaluate condition of cuttlefish hatchlings in ecologic and fisheries research. Thus, the present work could be seen as a new approach to the determination of standard nutritional condition patterns to be used when studying wild cuttlefish hatchlings condition. However, much work still has to be done concerning these indexes at different temperatures and under controlled conditions, to obtain some data that can be used as control to be applied.

According to Jackson and Choat (1992), short lifespan species display exponential or linear growth. Because of this, these animals spend about half of their life as small juveniles. Moltchanivskyj and Martinez (1998) added that this may not be a disadvantage to their survivorship, and that condition of these juvenile marine organisms, rather than size, may play an important role in population dynamics. According to Van Heukelem (1979), slow growth by under-nutrition results in individuals maturing at smaller sizes, whereas, if growth rates are reduced by lower temperatures, then maturation will occur at larger sizes. Based on this assumption, Moltchanivskyj and Martinez (1998) stated that the effects of temperature and nutrition on growth differ. Taking all this into account, laboratory determination of nucleic acid derived indexes patterns could provide some answers and clues about cuttlefish condition in the wild, and the associated recruitment. Nevertheless, they cannot be used as precise tools to evaluate cuttlefish condition during the first stages of their life, because they are not accurate enough.

ACKNOWLEDGMENTS

António Sykes and Pedro Domingues thank the Fundação para a Ciência e a Tecnologia, from the Portuguese government, that provided the funding for this research. This study was funded by CORRAM – "Cephalopoda: Octopodidae-relationship of the resource with the marine environment" Program Praxis XXI, ref. 2/2.1/MAR/1707/95.

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STUDY ON HERITABILITY OF GROWTH IN THE JUVENILE SEA URCHIN *STRONGYLOCENTROTUS NUDUS*

LIU XIAOLIN,^{1,3} CHANG YAQING,² XIANG JIANHAI,^{1,*} DING JUN² AND CAO XUEBIN²

¹Experimental Marine Biology Laboratory, Institute of Oceanology, CAS, Qingdao 266071, China; ²Key Lab of Mariculture and Biotechnology of the Ministry of Agriculture, Dalian Fisheries University, Dalian 116023, China; ³College of Animal Science and Technology, Northwest Sci-Tech University of Agriculture and Forestry, Yangling, Shaanxi 712100, China

ABSTRACT The heritability of growth of juvenile *Strongylocentrotus nudus* was analyzed using quantitative genetic methods. Twenty-one half-sib groups and 60 to 63 full-sib groups of juveniles were obtained by artificial fertilization of three to five females by single males based on a nested design. The body weight (g) and test diameter (cm) of the young was measured 3 and 5 months after metamorphosis. Maternal component estimates are significantly greater than paternal component estimates for both weight and diameter at both ages. Greater maternal components suggest large non-additive genetic effects that could not be differentiated with the available data. Estimates of heritability in the narrow sense calculated from the additive genetic component using a paternal half-sib correlation analysis ranged from 0.2167–0.4565 for weight and 0.2059–0.4998 for diameter. The results indicate significant maternal effects. The strength of the nested design and the paternal half-sib correlation analysis used in this study make the estimate the most precise and unbiased reported to date.

KEY WORDS: sea urchin, *Strongylocentrotus nudus*, growth, heritability

INTRODUCTION

Sea urchins are one of the most important aquaculture species in the world. The gonads have long been used as a luxury food and as a food source by common people in many countries (Hobson & Chave 1990, Shimabukuro 1991, Hagen 1996). Because of over-fishing, interest in aquaculture of sea urchins has increased greatly (Hagen 1998, Lawrence et al. 2001). One of the most important species for aquaculture is *Strongylocentrotus nudus* (Hagen 1996, Agatsuma 1998). Current culture of *S. nudus* has used seeds obtained from wild individuals (Gao & Chang 1999, Liao & Qiu, 1999). Analysis of populations of *S. nudus* in Japanese waters shows considerable range in size of small individuals, presumably of single cohorts (Agatsuma 1997). This could result from interaction of genetic characteristics and the environment. Vadas et al. (2002) found evidence for intrinsic variability in field populations of *Strongylocentrotus droebachiensis*. It is important to document the degree of heritability of growth in sea urchins because of its implications for both fisheries and aquaculture.

Demonstration of heritability is best done with comparisons of half-sib groups because they are less likely to be affected by environmental influence (Gjedrem 1992). Sib analysis techniques have been used for several important aquaculture species (Mallet et al. 1986, Rawson & Hilbish 1990, Hadley et al. 1991, Crenshaw et al. 1991, Newkirk et al. 1977, Benzie et al. 1997). The purpose of this study is to estimate heritability of growth in terms of body weight and diameter of juvenile *Strongylocentrotus nudus*.

MATERIALS AND METHODS

Experimental Design

This study used a classic nested mating design developed by Comstock and Robinson (1952) to partition the phenotypic varia-

tion in juvenile growth into its genetic and non-genetic causes. In this experiment each of 21 male *Strongylocentrotus nudus* was mated to 3 to 5 females, therefore generating 63 full-sib families and 21 half-sib families. The effects of males and females nested within males on growth were separated using nested analysis of variance (ANOVA). Juveniles were weighed and their diameters measured at 3 and 5 months of age.

Genetic Analysis

The covariance among full- and half-sibs provides the basis for the separation of phenotypic variance into genetic and environmental components of variance. The covariance among full- and half-sibs are calculated from the observed components of variance obtained from a three-level nested, unbalanced ANOVA (Table 1) and the General Linear Models procedure of the statistical analysis system (SAS) (Freund et al. 1986).

The experiment was a three-level classic nested, unbalanced design. Therefore the number of offspring in dams and in sires and in dams within sires should revise ("revise" means adjust). The effective means were computed using the equations:

Effective mean number of offspring in dams within sires

$$K_1 = [N - \sum(n_{ij}^2/dn_i)]/(D - S)$$

Effective mean number of offspring in dams:

$$K_2 = [\sum(n_{ij}^2/dn_i) - \sum(n_{ij}^2/N)]/(S - 1)$$

Effective mean number of offspring in sires:

$$K_3 = (N - \sum dn_i^2/N)/(S - 1)$$

in which S = number of sires, D = number of dams, n_{ij} = number of offspring of the i-th sire and j-th dam, dn_i = number of offspring of i-th sire, N = sum of number of offspring of all sires or all dams.

The phenotypic variance (V_p) was separated into the additive genetic variance (V_A), non-additive genetic variance (V_N) and environmental variance (V_E), and the environmental variance (V_E) was separated into the common environmental variance (V_{EC}) and the specific environmental variance (V_{ES}) using the standard sepa-

This is contribution number G1999012009 of 973 from the Chinese National Fundamental research project and Chinese High Technology Plan (2002AA628170).

*Corresponding author. E-mail: jhxiang@ms.qdio.ac.cn

TABLE 1.
Analysis of variance for components of phenotypic variation.

Source of Variance	Degree of Freedom (df)	Sum of Squares (SS)	Mean Square (MS)	Expected Mean Square E (MS)
Dams	F × M - 1	SS _M		
Sires	F - 1	SS _F	MS _F	$\sigma^2 + k_2 \sigma_M^2 + k_3 \sigma_F^2$
Dams/sires	F × (M - 1)	SS _{M(F)}	MS _{M(F)}	$\sigma^2 + k_1 \sigma_M^2$
Offspring (error)	F × M × (n - 1)	SS	MS	σ^2
Total	N - 1	SS _T		

ration of variance components (Falconer 1989). The causal components of variance were estimated from the full- and half-sib covariance using the relationships in Table 2.

Heritabilities were computed using the relationships:

$$h^2 = V_A / [V_A + V_{NA} + V_E]$$

Thus heritabilities in the narrow sense of paternal half-sib and maternal half-sib and full-sib were computed using the respective relationships:

$$\begin{aligned} h_{HS(S)}^2 &= 4 \times \sigma_S^2 / (\sigma_S^2 + \sigma_D^2 + \sigma^2) \\ h_{HS(D)}^2 &= 4 \times \sigma_D^2 / (\sigma_S^2 + \sigma_D^2 + \sigma^2) \\ h_{FS(D,S)}^2 &= 2 \times (\sigma_S^2 + \sigma_D^2) / (\sigma_S^2 + \sigma_D^2 + \sigma^2) \end{aligned}$$

Test of significant of heritability:

$$t = h^2 / \sigma_{h^2}$$

Paternal half-sib:

$$\begin{aligned} V_{(h^2)} &= \left(\frac{4}{\sigma_P^2} \right)^2 \left[V \left(\frac{MS_S}{k_3} \right) + V \left(\frac{MS_D}{k_3} \right) \right] \\ \sigma_{h^2} &= \frac{4}{\sigma_P^2} \sqrt{\frac{2}{k_3^2} \left[\frac{MS_S^2}{S-1} + \frac{MS_D^2}{N-D} \right]} \end{aligned}$$

Maternal half-sib:

$$\begin{aligned} V_{(h^2)} &= \left(\frac{4}{\sigma_P^2} \right)^2 \left[V \left(\frac{MS_D}{k_1} \right) + V \left(\frac{MS_E}{k_1} \right) \right] \\ \sigma_{h^2} &= \frac{4}{\sigma_P^2} \sqrt{\frac{2}{k_1^2} \left[\frac{MS_D^2}{D-S} + \frac{MS_E^2}{N-D} \right]} \end{aligned}$$

Full-sib:

$$\sigma_{h^2} = 2 \sqrt{\frac{2(1-r_{FS})^2[1+(k_1-1)r_{FS}]^2}{k_1(k_1-1)(D-1)}}$$

Experimental Animal

Collection and Maintenance

Parental *Strongylocentrotus nudus* were taken from a cultured population in Dalian Bay on the northern coast of the Yellow Sea on 10 September 2001. These individuals were held at 18–22°C under 500 × 1 illumination and fed *Laminaria japonica* ad libitum for 32 days before spawning on 12 October 2001.

Fertilization

Individuals were removed from the aquaria and allowed to drain for 30 minutes before 1 mL 0.5 M KCL was injected into the coelomic cavity via the peristomial membrane. They were placed on the tops of flasks filled with sea water and the eggs and sperm collected from each individual for 30–60 minutes.

The eggs of each female were fertilized with sperm from a single male. Approximately 150,000 eggs from each female were placed in 100-L containers. Sperm were diluted 1000-fold (Uehara et al. 1990) and a small amount of the diluted sperm was added to the eggs. Fertilization success was examined microscopically. The fertilized eggs were washed two to three times to remove excess sperm. The embryos were layered on the bottom of flasks and transferred into a 50-L container to develop at 17–21°C at a density of four to five individuals/mL. Normal plutei developed in 30–35 h. (Rahman et al. 2000, Rahman et al. 2001).

Rearing

The larvae were transferred to 100-L containers of filtered sea-water at 16°C. Densities of larvae from the 2-arm to the 8-arm stage were maintained at one to two individuals/mL. The water was changed twice daily. The larvae were fed *Chaetoceros gracilis*. Light was maintained at <300 LX. A small amount of air was bubbled into the water. After 3 months the juveniles from each fertilization group were placed separately into plastic cages suspended in a large pool. The juveniles were fed fresh *Laminaria*

TABLE 2.
Relationships between the covariance of full and half-sibs and causal components of phenotypic variance.

Component of Variance	Covariance Components	Causal Components	Calculation of Component of Variance
σ_F^2	COV _{HS}	1/4 V _A	{MS _F [(MS _{M(F)} - MS _E)/k ₁] × k ₂ - MS _E }/k ₃
σ_M^2	COV _{FS} - COV _{HS}	1/4V _A + 1/4V _{NA} + V _{EC}	(MS _{M(F)} - MS _E)/k ₁
σ_E^2	V _P - COV _{FS}	1/2 V _A + 3/4V _D + V _{ES}	MS _E
$\sigma_T^2 = \sigma_F^2 + \sigma_M^2 + \sigma^2$	V _P	V _A + V _{NA} + V _{EC} + V _{ES}	
$\sigma_F^2 + \sigma_M^2$	COV _{FS}	1/2 V _A + 1/4V _D + V _{EC}	

TABLE 3.

Body weight and test diameter of offspring at 3 and 5 months of age.

Growth Phase	Body Weight (g)		Test Diameter (mm)	
	Average	Standard Deviation	Average	Standard Deviation
3 months	0.014484	0.0103	2.916	0.945
5 months	1.366	0.377	8.492	2.841

japonica. The cages were changed every 2 months. The juveniles were weighed and their diameter measured at ages of 3 and 5 months.

RESULTS

Growth—Increase in Body-weight and Test Diameter

Mean and standard deviation of the increase in body weight and test diameter of offspring at 3 and 5 months of age are given in Table 3.

TABLE 4.

Analysis of variance for components of phenotypic variation of *Strongylocentrotus nudus* at 3 and 5 months of age.

Source of Variance	Body Weight			Test Diameter		
	Degrees of Freedom (df)	Mean Square (MS)	F-Value	Mean Square (MS)	F-Value	Expected Mean Square E (MS)
3 months						
Dam	62	4.08340×10^{-3}	9.893**	6.01406	6.737**	
Sire	20	7.26634×10^{-3}	17.605**	10.71717	12.006**	$\sigma^2 + k_2 \sigma_M^2 + k_3 \sigma_F^2$
Dams within sires	42	2.56772×10^{-3}	6.221**	3.77449	4.228**	$\sigma^2 + k_1 \sigma_M^2$
Full-sibs within dams	2210	4.12753×10^{-4}		0.89266		σ^2
Total	2272					
5 months						
Dams	59	0.79976	7.580**	69.16935	8.570**	
Sire	20	1.31728	12.485**	108.2978	13.418**	$\sigma^2 + k_2 \sigma_M^2 + k_3 \sigma_F^2$
Dams within sires	39	0.53436	5.065**	49.10348	6.084**	$\sigma^2 + k_1 \sigma_M^2$
Full-sibs within dams	2045	0.10551		8.07108		σ^2
Total	2104					

**P < 0.01; S = sires; D = dams; K_1 is the weighed mean offspring number of females. K_2 is the weighed offspring number of females within sire. K_3 is the weighed mean offspring number of sires.

TABLE 5.

Relationships between the covariance of full and half-sibs and causal components of phenotypic variance.

Component of Variance	Causal Components	Covariance Components	Result of Component of Variance			
			3 Month		5 Month	
			Body Weight	Test Diameter	Body Weight	Test Diameter
σ_F^2	$1/4 V_A$	Cov_{HS}	4.13895×10^{-5}	0.06027	0.00678	0.50447
σ_M^2	$1/4 V_A + 1/4 V_{NA} + V^{EC}$	$\text{Cov}_{FS} - \text{Cov}_{HS}$	6.16974×10^{-5}	0.08308	0.01280	1.22465
σ^2	$1/2 V_A + 3/4 V_D + V_{EW}$	$V_P - \text{Cov}_{FS}$	4.275×10^{-4}	0.89264	0.10551	8.07108
$\sigma_P^2 = \sigma_F^2 + \sigma_M^2 + \sigma^2$	$V_A + V_{NA} + V_{EC} + V_{EW}$	V_P	5.40587×10^{-4}	1.03598	0.12509	9.80020
$\sigma_F^2 + \sigma_M^2$	$1/2 V_A + 1/4 V_D + V_{FC}$	COV_{FS}	1.03087×10^{-4}	0.14335	0.01958	1.72912

TABLE 6.

Heritabilities in narrow sense (h^2) and standard error (σ_h^2).

	3 Months of Age		5 Months of Age	
	Body Weight	Diameter	Body Weight	Diameter
h_S^2	$**0.306 \pm 0.055$	$**0.233 \pm 0.046$	$**0.217 \pm 0.042$	$**0.206 \pm 0.041$
h_D^2	$**0.457 \pm 0.071$	$**0.321 \pm 0.046$	$**0.409 \pm 0.064$	$**0.500 \pm 0.072$
h_{FS}^2	$**0.381 \pm 0.063$	$**0.277 \pm 0.051$	$**0.313 \pm 0.054$	$**0.353 \pm 0.058$

**Denotes very significance between h^2 with zero ($P < 0.01$); $t_{0.01, \infty} = 2.576$.

Analysis of Variance of Body Weight and Test Diameter of Offspring

Analysis of variance demonstrated great differences in body weight and diameter of juveniles from different females mated with the same male and between males at both 3 and 5 months of age (Table 4).

Effective mean number of offspring for sires and dams after 3 months was computed as follows: effective mean number of offspring in dams within sire is $K_1 = 34.689$, in dams is $K_2 = 38.821$, and in sires is $K_3 = 107.719$. Effective mean number of offspring for sires and dams after 5 months was computed as follows: $K_1 = 33.505$; $K_2 = 36.831$; $K_3 = 109.268$.

The causal components of variance were estimated from the full- and half-sib covariance using the relationships in Table 5.

Estimations of Heritability of Body Weight and Test Diameter of Offspring

Heritabilities in the narrow sense of paternal half-sib and maternal half-sib and full-sib of body weight and diameter of 3-month-old *Strongylocentrotus nudus* were calculated on the result of component of variance and test ($t = h^2/\sigma_h^2$) of significant of heritability respectively as Table 6.

All of the heritability in the narrow sense of sire half-sib and the heritability in the broad sense of dam full-sib of body weight, test diameter of *S. intermedius* were significantly different from zero (t -test, $P < 0.01$).

Estimated heritability was somewhat different among the sire heritability, dam heritability, and the pooled (combined) heritability for all three traits. The estimates of dam heritability were higher than that of sire heritability, and the pooled (combined) heritability was moderate.

DISCUSSION

Predicted heritabilities of larval growth have been reported for full-sib correlation analysis for *Crassostrea virginica* (Lannan 1972, Haley et al. 1975, Newkirk et al. 1977) and *Penaeus vannamei* (Carr et al. 1997) and half-sib correlation analysis for *Mercenaria mercenaria* (Rawson & Hilbish 1990), *Macrobrachium rosenbergii* (Malecha et al. 1984), *Penaeus vannamei* (Carr et al. 1997, Benzie et al. 1996), and *Penaeus stylirostri* (Benzie et al. 1996). Predicted heritabilities of shell traits in wild littorina saxatilis populations have been reported for full-sib correlation analysis and offspring-mother regression (Carballo et al. 2001). Estimates of heritabilities in the narrow sense generally ranged from 0.2–0.7. Realized heritability for increase in rate of growth in northern quahog and *Argopecten irradians concentricus* (Crenshaw et al. 1996, Crenshaw et al. 1991) and realized heritability estimates for growth in the Chilean oyster *Ostrea chilensis* (Toro et al. 1995). In our study, the estimates of heritabilities in the narrow sense for

body weight at 3–5 months of age ranged from 0.217–0.457, consistent with those reported for other species. However, estimates are based on full-sib families bias heritabilities upwards when dominance and maternal effects are present (Lester, 1988). Because of the nested design and a paternal half-sib correlation analysis used, the estimate reported here is more precise and unbiased. This is the first report of heritability in the narrow sense reported for sea urchins.

An animal model includes a random effect for the additive genetic effect of each individual; and incorporates a complete set of additive genetic relationships among all individuals; and allows an unbiased estimation of variance components, even for the data involving selection and non-random mating (Gall & Bakar 2002, Sorensen & Kennedy 1986, Su et al. 1997). In this investigation full-sib family was taken as a random effect in a simple random model to account for the covariance among full-sibs caused by common environmental, dam and non-additive genetic effects, and half-sib family was used to account for covariance among half-sibs caused by common environmental, dam, sire, and non-additive genetic effects. The results from the analyses based on this model were expected to be unbiased estimates of genetic parameters for the base population.

The much larger heritabilities computed from the female additive genetic component indicate the female genetic component still contain common environmental effects, maternal effects or non-additive genetic variance in the body weight or diameter. Dam effects are omnipresent in the study. The fact that the juvenile phase may indicate that the quality of yolk reserves plays a role in early development. Dam effects may persist after the onset of exogenous feeding. Crandell and Gall (1993) reported that dam effects persist up to 2 years in rainbow trout and up to 18-month-old Arctic char (Nilsson 1994).

The estimated heritability indicates significant additive genetic variation for body weight and test size all over the sampling periods. Sire heritability for the different variables were lower than dam heritability in many cases. However, all heritabilities in the narrow sense of body weight, test diameter of *S. intermedius* were significantly different from zero ($P < 0.01$). The heritabilities in the narrow sense estimate obtained from this experiment indicate there is sufficient variation in the base population of sea urchins to respond to natural or artificial selection on juvenile growth weight. This justifies selection of juveniles based on growth characteristics for cultured brood stock.

ACKNOWLEDGMENTS

The authors thank J. Song, G. D. Wang, R. L. Xing, and S. G. Yan for their invaluable assistance in the laboratory production of the family lines. They also thank J. M. Lawrence for editing the paper.

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A SET OF TESTS FOR THE PHENOTYPIC IDENTIFICATION OF CULTURABLE BACTERIA ASSOCIATED WITH GALICIAN BIVALVE MOLLUSC PRODUCTION

J. A. GUISANDE, M. MONTES, R. FARTO, S. P. ARMADA, M. J. PÉREZ AND T. P. NIETO*

Área de Microbiología, Departamento de Biología Funcional y Ciencias de la Salud, Facultad de Ciencias, Universidad de Vigo, Vigo, Spain

ABSTRACT To select a set of biochemical tests that provide a rapid identification of culturable bacteria associated with Galician bivalve molluscs cultures, the phenotypic diversity of some bacterial species was investigated. A total of 488 strains isolated from water, phytoplankton, larva, seed and reproductive phases in oysters and clams cultures in Galicia (NW of Spain) and 51 type and reference strains were characterized by 92 physiologic, morphologic and biochemical tests. The 80% of isolates were facultative anaerobes and the remaining 20% were aerobes. Using numerical taxonomy and probabilistic methods, 57% of facultative anaerobic isolates could be identified: *Vibrio aestuarii*, *V. splendidus* biotype 1, *V. alginolyticus*, *V. mytili*, *V. (Listonella) anguillarum*, *V. salmonicida*, *V. furnissii* and *V. orientalis*. The predominant aerobic strains were *Shewanella* spp., *Pseudoalteromonas* spp., *Pseudomonas* spp. and *Alcaligenes denitrificans*. *Vibrio* was the most frequently isolated genus from oysters, clams and their culture water. *Pseudomonas* and *Pseudoalteromonas* were the main isolated genus from phytoplankton samples. A selection of a set of tests for the phenotypic and presumptive identification of the main culturable bacterial groups was possible. Since this set of tests provides a rapid identification, it will be very useful in the quality control of bivalve mollusks, in industrial treatment plants or in the work of hygiene and health control staff.

KEY WORDS: oysters, clams, presumptive bacterial identification, aerobic bacteria, facultative anaerobic bacteria, *Vibrio*, numerical taxonomy

INTRODUCTION

The culture of bivalve molluscs represents the 8% of the world fish production and the 26% of the world aquaculture production. Bivalve mollusk production has been increasing in the last 50 years worldwide, with a triplication of its production in the last decade (Lovatelli 2003). Spain is the main bivalve mollusks producer in Europe, with an annual production of 275,000 tons, where the main culture volume is concentrated in Galicia (NW of Spain).

The development of modern techniques allows the maintenance of cultures with a high density of mollusks, making intensive cultures possible but also increasing the risks and consequences of infectious outbreaks.

The association of microorganisms with animal tissues can be symbiotic or pathogenic or, as in the case of mollusks, coincidental with the process of filter feeding. The great phenotypic diversity of these bacterial strains makes identification of isolates difficult. Also, certain bacterial species have been described as pathogenic to bivalve mollusks and/or to people who consume them. These species include: *V. tapetis* (Paillard et al. 1989, Castro et al. 1993, Castro 1994, Robledo et al. 1994, Borrego et al. 1996), *V. tubiashii* (Tubiash et al. 1965), *V. vulnificus* (Tracket et al. 1984, Horré & Günter 1996), *V. (Listonella) anguillarum* (Grischowsky & Liston 1974, Elston 1989, Montilla et al. 1994), *V. fluvialis* (Lee et al. 1981), *V. pelagius* (Montilla et al. 1994), *V. mimicus* (Mattè et al. 1994), *Vibrio cholerae* (Kaper et al. 1983), *V. parahaemolyticus* (Kaper et al. 1983), *V. alginolyticus* (Grischowsky & Liston 1974, Elston 1989), or *Plesiomonas shigelloides* (Prescott et al. 1999).

The aim of this study is to identify the bacteria associated with the culture of bivalve molluscs in Galicia to select tests that can be useful to separate environmental bacteria species associated with mollusks cultures and the most frequent potentially pathogenic strains. Such a set of tests will be very useful for undertaking the

quality control of bivalve mollusks, in industrial treatment plants or in the work of hygiene and health control staff.

MATERIALS AND METHODS

Bacterial Strains

Samples were obtained from different stages (seed, larval, and reproductive) in cultures of clams (*Ruditapes decussatus*, *Venerupis pullastra*, *Tapes japonica*) and oysters (*Ostrea edulis*), as well as from the surrounding water and phytoplankton. All animals were washed first to remove loosely associated bacteria and contaminants. All sterilizations were done at 121°C for 15 min and all filtrations through 0.22-µm pore size porosity filters. Reproductive bivalve mollusks (50 g of meat and intervalval liquid) were homogenized in 100 mL of sterile marine phosphate-buffered saline (PBS) (20 g of NaCl per liter, 0.2 g of KCl per liter, 1.15 g of PO₄HNa₂ per liter, 0.2 g of PO₄H₂K per liter; pH 7.1 to 7.2). Bivalve mollusks in the larval and seed stages (50 units each) were separately homogenized in 100 mL of PBS. Phytoplankton samples were obtained through the filtration of 1000 mL of water and their suspension in 100 mL of PBS. Serial dilutions (1:10; 1:100; 1:1000) of all these samples were plated in duplicate on Marine Agar (MA, Cultimed, Barcelona, Spain) and Thiosulphate-Citrate-Bile-Sucrose (TCBS, Cultimed), including water samples. Plates were incubated at 22°C for 4 days. Five colonies of each morphology were picked off and streaked on MA to obtain pure cultures, which were inoculated on Nutritive Broth (Cultimed) with 2% (w/v) NaCl (Panreac, Barcelona, Spain) and 15% (v/v) of glycerol (Panreac) for their conservation at -80°C.

Sampling was carried out over 12 consecutive months (1 sample a month) in bivalve mollusks culture systems located on the Galician coasts at: Bueu, O Grove, Ribadeo, Couso, Malpica, and Vilagarcía de Arousa. According to these methods, 480 strains were isolated.

The following 51 type and reference strains were included in this study for comparative purposes and were characterized under the same conditions and using the same tests as for the remainder

*Corresponding author. E-mail: mtperez@uvigo.es

of the isolates: *Agrobacterium ferrugineum* CECT (Colección Española de Cultivos Tipo, Valencia, Spain) 4356, *Ag. sanguineum* CECT 4271^T, *Ag. stellulatum* CECT 4269, *Alcaligenes denitrificans* CECT 449^T, *Halomonas aquamarina* CECT 5000^T, *Marinomonas communis* CECT 5003^T, *M. vaga* CECT 5004¹, *Photobacterium angustum* CECT 4193^T, *Ph. damsela* subsp. *damsela* ATCC (American Type Culture Collection, Manassas, VA, USA) 33539¹, *Ph. damsela* subsp. *piscicida* ATCC 17911, *Ph. phosphoreum* CECT 4172, *Pseudoalteromonas citrea* CECT 575^T, *Pseudoalteromonas espejiana* CECT 5002^T, *Pseudoalteromonas haloplanktis* CECT 4188^T, *Pseudoalteromonas undina* CECT 5006¹, *Pseudomonas fluorescens* CECT 378¹, *Ps. nautica* CECT 5005^T, *Ps. putida* CECT 324^T, *Shewanella hanelai* CECT 5194^T, *Vibrio aestuarii* ATCC 35048¹, *V. albensis* LMG (Laboratoire de Microbiologie, Université de Gent, Belgium) 4406 (*V. cholerae*, Garrity et al. 2002), *V. alginolyticus* CECT 521^T, *V. anguillarum* 43-F, NCIMB (National Collections of Industrial and Marine Bacteria, Aberdeen, UK) 571 and NCIMB 6 (*Listonella anguillarum*, Garrity et al. 2002), *V. campbellii* CECT 523^T, *V. carchariae* LMG 7890^T (*V. harveyi*, Garrity et al. 2002), *V. costicola* LMG 6460 (*Salinivibrio costicola* subsp. *costicola*, Garrity et al. 2002), *V. diazotrophicus* LMG 7893^T, *V. fischeri* LMG 4414^T, *V. fluvialis* LMG 7984^T, *V. furnissii* LMG 7910¹, *V. harveyi* LMG 4044^T, *V. lentus* CECT 5110, *V. metschnikovii* ATCC 7708, *V. mimicus* LMG 7896^T, *V. mytili* CECT 632¹, *V. natriegens* LMG 10935¹, *V. nereis* LMG 3895^T, *V. nigripulchritudo* CECT 628^T, *V. ordalii* CECT 582^T, *V. orientalis* CECT 629^T, *V. parahaemolyticus* LMG 2850^T, *V. pelagius* CECT 4202^T (*Listonella pelagia*, Garrity et al. 2002), *V. proteolyticus* LMG 3772^T, *V. salmonicida* ATCC 43839¹, *V. scophthalmi* AO89^T, *V. splendidus* biotype I ATCC 33125^T, *V. tapetis* CECT 4600^T, *V. tubiashii* LMG 10936^T, and *V. vulnificus* biotype II CECT 897.

Phenotypic Characterization

Bacterial strains were characterized by 92 physiologic, morphologic, and biochemical tests. Cultures grown during 24 h at 22°C on Tryptic Soy Agar (TSA, Cultimed) and supplemented up to 2% (w/v) NaCl (TSA 2%) were used as inocula. Unless otherwise indicated, all incubations were undertaken at 22°C, commercial media were supplemented up to 2% (w/v) NaCl and results were read after 48 hours.

All tests and methods were carried out as described by Montes et al. (1999), except that the hydrolysis of urea was tested on Stuart's Urea Broth (Pronadisa, Madrid, Spain) and the Gram character was performed using the KOH 3% (v/v) (Analema, Vigo, Spain) method (Buck 1982).

All tests were assayed on plates and were spot inoculated with a multipoint inoculator, except for O/F (oxidative or fermentative metabolism of glucose), ODC (ornithine decarboxylase), LDC (lysine decarboxylase), HDC (histidine decarboxylase), ADH (arginine dihydrolase), Voges-Proskauer, methyl red, Kligler, nitrate reduction, indole production, and the hydrolysis of urea when tests were performed in tubes.

Coding of Data

Results were coded using the following criteria: 1 for positive results, 0 for negative results and 9 for non-comparable or missing values. Data were processed with the NTSYS-pc, version 1.8 (Rohlf 1994). Similarity matrices were calculated using the Simple Matching coefficient (S_{SM}) and Jaccard's coefficient (S_J). Phenotypes

were clustered using the Unweighted Pair Group Method, with Arithmetic mean (UPGMA). The correlation between the dendrogram and each similarity matrix (cophenetic correlation) was determined using the cophenetic correlation coefficient (r).

The reproducibility of the tests was evaluated by analyzing 10% of strains in duplicate, as suggested by Sneath and Johnson (1972).

For phenotypes that could not be identified by numerical taxonomy methods, probabilistic identification was performed with the "Probabilistic identification of bacteria" program, version 1.10 (Bryant 1995). This computer program includes the data matrices of Bryant et al. (1986) and Alsina and Blanch (1994a), providing the most suitable probabilistic identification of each phenotype.

RESULTS

Numerical Taxonomy

Seventy-six per cent of a total of 488 strains were isolated in MA and the remaining 24% in TCBS. Some 80% of isolates were Gram negative, oxidase positive and with the ability to ferment glucose (facultative anaerobic strains). The remaining 20% were Gram negative, oxidase positive, and non-fermentative strains (aerobic strains).

The probability of an erroneous result was $P = 2.596\%$ for aerobic strains and $P = 2.045\%$ for facultative anaerobic strains. According to the criteria suggested by Sneath and Johnson (1972), each of these values and their average (2.3078%) are acceptable values (lower than 5%) and are indicative of good reproducibility.

Five tests were deleted from the data matrices because of their variances (higher than 0.1, according to Bryant et al. 1986): acid production from arbutine, use of aspartate or alginate as sole carbon source, production of lipase using tween 20 as substrate and β -hemolysis.

Furthermore, 9 tests giving the same result were also eliminated for aerobic strains: growth at 22°C (+: positive result), growth at pH 7.5 (+), oxidase (+), histidine decarboxylation (-: negative result), glucose fermentation (-), Voges-Proskauer test (-), acid production from inositol (-) or L-rhamnose (-), and the Gram character, that was negative. For facultative anaerobic strains, 9 tests were deleted from the data matrix, as they had always shown the same result: growth at 22°C (+), growth at pH 4.5 (-), oxidase (+), glucose fermentation (+), glucose on Kligler (+), acid production from inositol (-) or raffinose (-), motility (+) and the Gram character, that was negative.

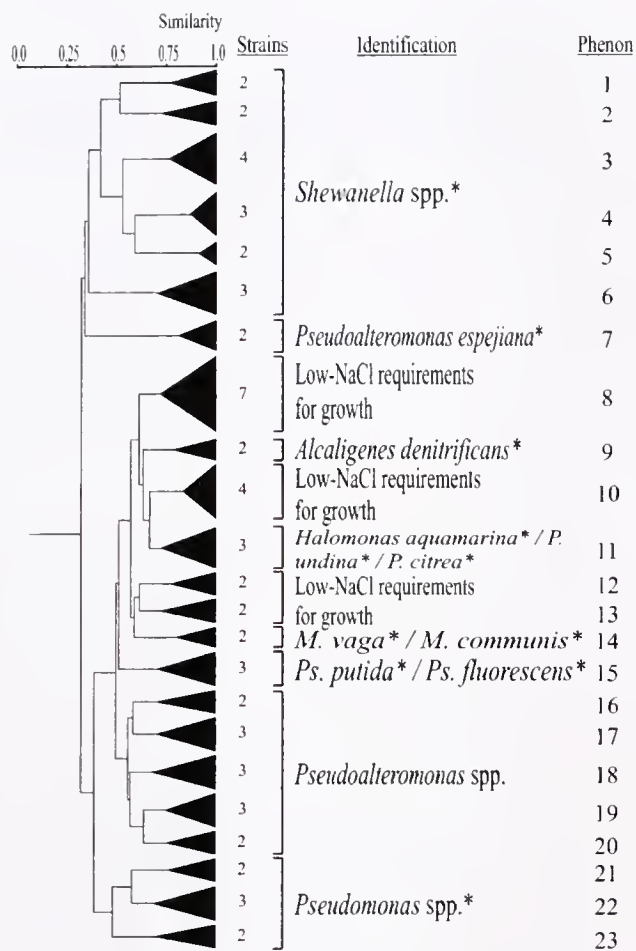
For the aerobic strains, the number of final matrices was 106 (strains) \times 78 (tests) and for facultative anaerobic strains the number was 434 (strains) \times 78 (tests).

Similarities were calculated using the S_{SM} and S_J coefficients. Phenotypes were clustered using the UPGMA. Cophenetic correlation showed the best fit (Rohlf 1994) using the S_J coefficient ($r = 0.81621$ for aerobic strains and $r = 0.80555$ for facultative anaerobic strains).

This study focuses on clustered isolates: 57% of aerobic strains (with a S_J /UPGMA similarity level of 69%) and 88% of facultative anaerobic strains (with a S_J /UPGMA similarity level of 69%).

Aerobic Strains

A phenotypic dendrogram constructed using the S_J /UPGMA analysis included 23 phenotypes (Fig. 1) with a similarity level of 69%, clustering 57% of aerobic isolates. Only 4 isolates were identified:



*: A reference strain was included within as identified group

Figure 1. Simplified dendrogram of aerobic strains showing the phena defined with a value of $S_j \geq 69\%$.

Shewanella hanedai (one isolate), *Alcaligenes denitrificans* (one isolate), *Pseudoalteromonas espejiana* (one isolate) and *Pseudomonas nautica* (one isolate).

Independently of the S_j similarity level and due to the great diversity of unidentified aerobic isolates that was found, five different main groups were defined (see Fig. 1) taking shared characteristics of close phena into account: *Shewanella* spp. (16% of clustered isolates), low-NaCl requirements for growth (16% of isolates), *Pseudoalteromonas* spp. (15% of isolates), *Pseudomonas* spp. (8% of isolates), and *Alcaligenes denitrificans* (1% of isolates).

In addition to the tests for which the same result was obtained, as described earlier for all aerobic strains, clustered aerobic isolates always displayed the same response for the following tests: growth at 28°C (+), pH 4.5 (-), pH 9 (+), growth in 3.5% (w/v) NaCl (+), H_2S production on TCBS (-), catalase reaction (+), ODC and LDC (-); oxidative character on O/F medium (+), utilization of glucose or lactose on Kligler medium (-), methyl red reaction (-), indole production (-), α -haemolysis (-), acid production from amygdaline, L-arabinose, D-cellobiose, ethanol, D-fructose, D-galactose, glycerol, lactose, D-mannitol, D-melibiose, D-raffinose, ribose, sucrose, salicin, sorbitol or D-trehalose (-); degradation of urea, chitin, gelatin, casein, DNA, lecithin, chondroitin, cellulose

or agar (-); utilization of citrate as sole carbon source (-), and motility (+).

Differential phenotypic characteristics are given in Table 1. The main characteristics that allow different groups to be defined are cited below:

Shewanella spp.

This group was constituted by phenon 1 (two isolates), phenon 2 (two isolates), phenon 3 (four isolates), phenon 4 (three isolates), phenon 5 (one isolate and the reference strain of *Shewanella hanedai*), and phenon 6 (three isolates). All showed a mesophilic character (growth between 10°C and 28°C) and had NaCl requirements for growing, despite being unable to tolerate high NaCl concentrations (7% or even 5% in some cases). Half of them were able to produce H_2S on Kligler medium, and most grew on TCBS medium and carry out nitrate reduction, as well as growth maintenance at pH 10 and sensitivity to tetracycline (30 µg/disc). They were generally unable to use any of the assayed substrates as sole carbon source.

Low-NaCl Requirements for Growth

This group included phenon 8 (7 isolates), 10 (4 isolates), 12 (2 isolates), and 13 (2 isolates). The main characteristics of this group are growth maintenance in low NaCl concentrations (0.5%), which indicates the low NaCl requirements for growth, as well as NaCl tolerance of up to 7%, growth maintenance at pH 10 and the use of different substrates as sole carbon source: acetate, β -alanine, DL-alanine, L-arginine, L-lysine, malonate, L-proline, propanol, pyruvate, L-serine or succinate. All had sensitivity to tetracycline (30 µg/disc).

Pseudoalteromonas spp.

This group included the following phena: 7 (1 isolate and the reference strain of *Pseudoalteromonas espejiana*), 16 (2 isolates), 17 (3 isolates), 18 (3 isolates), 19 (3 isolates), and 20 (2 isolates). A reference strain of *Pseudoalteromonas haloplanktis* was clustered close to phenon 20. All these strains showed NaCl requirements for growth, tolerating most of them NaCl concentrations of up to 7%. These strains were able to grow at 28°C (but not usually at 37°C) and to carry out starch degradation. Glycine and L-proline were the sole substrates they could use as carbon sources.

Pseudomonas spp.

This group was constituted by phenon 15 (one isolate was clustered with reference strains of *Pseudomonas putida* and *Pseudomonas fluorescens*), phenon 21 (2 isolates), phenon 22 (2 isolates) were clustered with a reference strain of *Pseudomonas nautica*, and phenon 23 (2 isolates). Most of them were able to grow between 10°C and 28°C (even at 44°C in some cases), at pH 10 and had low-NaCl requirements for growth (0.5% NaCl). They were able to use acetate, L-proline and pyruvate as sole carbon sources and were sensitive to tetracycline (30 µg/disc).

Alcaligenes denitrificans

One bacterial isolate was clustered with the reference strain of *Alcaligenes denitrificans* (phenon 9). Both were able to grow between 10°C and 37°C and at pH 10; showed low-NaCl requirements for growth and were not able to tolerate high NaCl concentrations (7% or even 5% in some cases). They were able to carry out nitrate reduction and the use of different substrates as sole carbon source, such as acetate, β -alanine, DL-alanine, glycine,

TABLE 1.
Differential characteristics of groups identified as containing aerobic isolates.

Test	Group 1 N = 16		Group 2 N = 15		Group 3 N = 15		Group 4 N = 10		Group 5 N = 2	
ADH	12.5	(-)	0	-	0	-	40	v	0	-
Glucose oxidation	0	-	6.7	-	0	-	40	v	0	-
K1A/H ₂ S	50	v	0	-	0	-	0	-	0	-
Nitrate reduction	93.7	+	53.3	v	13.3	(-)	70	(+)	100	+
Growth at:										
4°C	62.5	v	66.7	v	26.7	(-)	30	v	0	-
10°C	100	+	93.3	+	66.7	v	80	(+)	100	+
37°C	0	-	46.7	v	20	(-)	50	v	100	+
44°C	0	-	6.7	-	0	-	40	v	0	-
pH 10	100	+	86.7	(+)	46.7	v	100	+	100	+
Growth in:										
0.5% NaCl	6.2	-	93.3	+	13.3	(-)	80	(+)	100	+
5% NaCl	43.7	v	100	+	100	+	100	+	50	v
7% NaCl	0	-	100	+	93.3	+	60	v	0	-
10% NaCl	0	-	33.3	v	46.7	v	40	v	0	-
Crystal violet	18.7	(-)	6.7	-	0	-	30	(-)	50	v
TCBS Agar	81.2	(+)	0	-	0	-	20	(-)	0	-
TCBS (yellow)	37.5	v	0	-	0	-	0	-	0	-
Acid from:										
D-galactose	0	-	7.7	-	ND	ND	33.3	v	0	-
D-mannose	13.3	(-)	13.3	(-)	ND	ND	33.3	v	0	-
Degradation of:										
Starch	43.7	v	6.7	-	80	(+)	40	v	0	-
Esculin	75	(+)	46.7	v	41.7	v	10	-	0	-
Use as sole carbon source:										
acetate	18.7	(-)	93.3	+	60	v	90	+	100	+
β-alanine	0	-	86.7	(+)	13.3	(-)	40	v	100	+
DL-alanine	18.7	(-)	93.3	+	60	v	30	(-)	100	+
L-arginine	0	-	80	(+)	60	v	50	v	50	v
Glycine	6.2	-	46.7	v	73.3	(+)	40	v	100	+
Inulin	0	-	46.7	v	0	-	10	-	0	-
L-lysine	0	-	100	+	26.7	(-)	30	(-)	100	+
Malonate	0	-	93.3	+	6.7	-	30	(-)	100	+
L-phenylalanine	0	-	46.7	v	53.3	v	40	v	100	+
L-proline	6.2	-	100	+	80	(+)	90	+	100	+
Propanol	18.7	(-)	80	(+)	26.7	(-)	50	v	100	+
Pyruvate	37.5	v	100	+	66.7	v	70	(+)	100	+
L-serine	6.2	-	73.3	(+)	66.7	v	50	v	100	+
Succinate	18.7	(-)	73.3	(+)	46.7	v	60	v	50	v
L-tartrate	0	-	46.7	v	0	-	20	(-)	50	v
L-tryptophan	0	-	53.3	v	6.7	-	30	(-)	50	v
Uracil	0	-	53.3	v	13.3	(-)	10	-	0	-
Sensitivity to:										
O/129 (150 µg)	50	v	35.7	v	ND	ND	ND	ND	0	-
Tetracycline (30 µg)	85.7	(+)	100	+	ND	ND	100	+	100	+

Group 1.-*Shewanella* spp.; Group 2.-Low-NaCl requirements for growth; Group 3.-*Pseudoalteromonas* spp.; Group 4.-*Pseudomonas* spp.; Group 5.-*Alcaligenes denitrificans*.

Discriminatory tests for the biochemical identification of strains isolated from bivalve molluscs that are Gram negative, motile, oxidase positive, aerobic and able to grow at 22°C strains are expressed in bold.

Criteria (data are expressed in percentage of positive results): ND: No data; +: Positive result ($\geq 90\%$ of positive results); -: Negative result ($\leq 10\%$ of positive results); (+): Mainly positive results ($\geq 70\% < 90\%$ of positive results); (-): Mainly negative results ($\geq 10\% < 30\%$ of positive results); v: Variable results ($> 30\% < 70\%$ of positive results).

L-lysine, malonate, L-phenylalanine, L-proline, propanol, pyruvate, or L-serine. They were sensitive to tetracycline (30 µg/disc), but not to O/129 (150 µg/disc).

The following 6 tests (Table 1) are proposed as discriminatory and useful for the biochemical identification of Gram negative, motile, oxidase positive, aerobic, and able to grow at 22°C strains associated with the culture of Galician bivalve molluscs: growth in

different concentrations of NaCl (0.5% and 7%), nitrate reduction, growth on TCBS medium and the use of DL-alanine or malonate as sole carbon source.

Facultative Anaerobic Strains

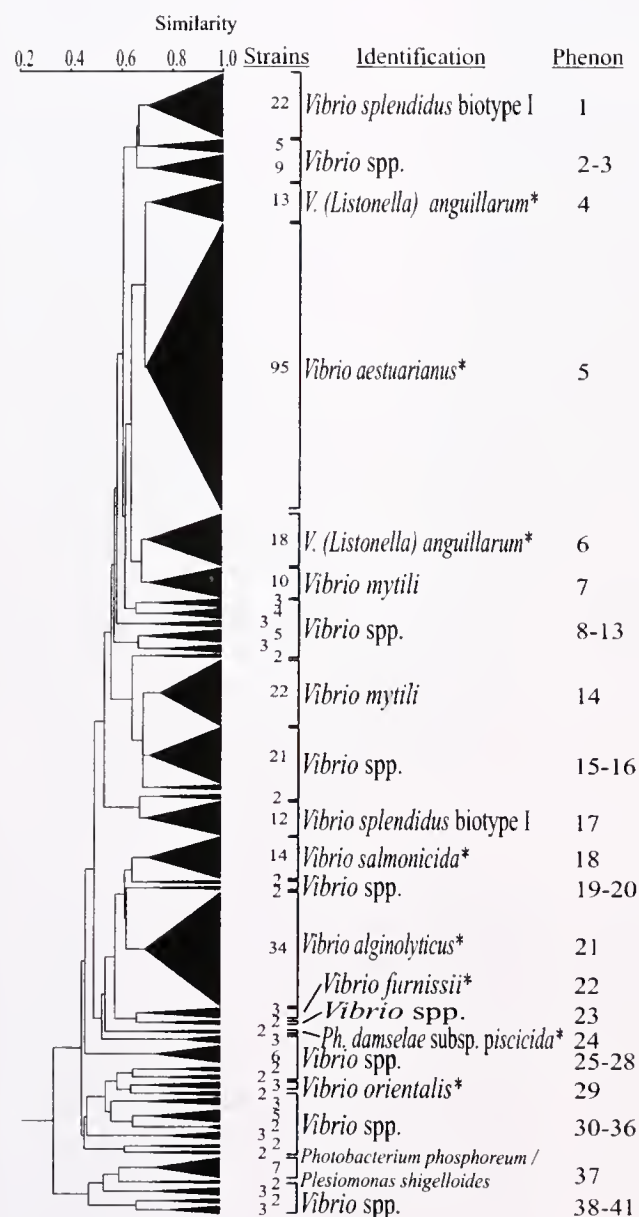
A dendrogram was obtained applying Jaccard's similarity coefficient and using the UPGMA as a clustering method. In total, 41

phena (clustering 88% of facultative anaerobic strains) were established with a S_J /UPGMA similarity level of 69% (Fig. 2). The inclusion of reference strains allowed the identification of eight phena: *Vibrio aestuarianus* (22% of isolates), *V. alginolyticus* (8% of isolates), *V. (Listonella) anguillarum* (7% of isolates), *V. salmonicida* (3% of isolates), *V. furnissii* (1% of isolates), *V. orientalis* (1% of isolates), and *Photobacterium damsela* subsp. *piscicida* (1% of isolates). Five phena were identified as *V. splendidus* biotype I (8% of isolates), *V. mytili* (7% of isolates) and *Photobacterium phosphoreum*/*Plesiomonas shigelloides* (2% of isolates), showing a probability of identification of over 75%, according to the data matrices of Bryant et al. (1986) and Alsina and Blanch (1994a) ("Probabilistic identification of bacteria" program, version 1.10, Bryant, 1995). Some 59% of all Gram negative, facultative anaerobic, motile and oxidase positive isolates could be

identified by these methods. The use of updated keys for biochemical identification of *Vibrio* species (Alsina & Blanch 1994b) did not provide an improved identification. The remaining 28 unidentified phena were assigned as "*Vibrio* spp."

In addition to the tests for which the same result was obtained, as described earlier for all facultative anaerobic strains, clustered facultative anaerobic isolates always displayed the same response for the following tests: growth at pH 7.5 (+), pH 9 (+), pH 10 (+), H_2S production on TCBS (-), HDC (-), utilization of lactose in Kligler medium (-), H_2S production in Kligler medium (-), Voges-Proskauer reaction (-), methyl red reaction (+), acid production from amygdaline, L-arabinose, ethanol, lactose, D-melibiose, L-rhamnose, salicin or sorbitol (-), degradation of urea or cellulose (-), and sensitivity to tetracycline (30 μ g/disc) (+).

For each identified group (Table 2), in keeping with previous description by other authors and in addition to the responses already mentioned for all facultative anaerobic strains, the main characteristics were:



*: A reference strain was included within an identified group

Figure 2. Simplified dendrogram of facultative anaerobic strains showing the phena defined with a value of $S_J \geq 69\%$.

V. aestuarianus

This group was constituted by phenon 5 (94 isolates and a reference strain of *V. aestuarianus*). Strains included in this phenon showed a negative result for the following tests: lysine and ornithine decarboxylation, growth at 37°C and in 10% NaCl. However, they showed a positive result for Thornley's arginine dihydrolase test, growth at 28°C, nitrate reduction, starch degradation, acid production from D-mannitol, use of citrate as sole carbon source, and susceptibility to O/129 (150 μ g/disc).

V. splendidus biotype I

This group included phenon 1 (22 isolates) and phenon 17 (12 isolates). These bacterial strains were unable to grow at 37°C, in 10% NaCl or in crystal violet. They were unable to use β -alanine, inulin, L-lysine, malonate, L-phenylalanine, propanol, or L-tryptophan as sole carbon source. They showed negative responses for ornithine or lysine decarboxylase tests, and positive results for acid production from D-cellobiose, D-mannitol and ribose, use of L-proline as sole carbon source, and growth at 28°C.

V. alginolyticus

This group was formed by phenon 21 (33 isolates and a reference strain of *V. alginolyticus*). Isolates included in this phenon showed a negative result for Thornley's arginine dihydrolase test and use of propanol as sole carbon source. However, they were able to carry out nitrate reduction, hydrolysis of casein, indole production, growth on 10% NaCl and at 44°C, lysine decarboxylation, acid production from sucrose or D-trehalose, use of acetate, β -alanine, DL-alanine, L-arginine or citrate as sole carbon source. They showed positive results for catalase test and a variable result for ornithine decarboxylation.

V. mytili

This group was constituted by phenon 7 (10 isolates) and phenon 14 (22 isolates). These strains showed negative responses in tests for growth at 37°C, lysine or ornithine decarboxylation, hydrolysis of DNA or gelatin and acid production from D-mannose. They showed a positive result for Thornley's arginine dihydrolase test and acid production from sucrose.

TABLE 2.
Differential characteristics of phena identified as containing facultative anaerobic isolates.

Test	Phenon 1 N = 22		Phenon 4 N = 13		Phenon 5 N = 95		Phenon 6 N = 18		Phenon 7 N = 10		Phenon 14 N = 22	
Catalase	100	+	92.3	+	34.7	v	94.4	+	90	+	13.6	(-)
ADH	95.5	+	100	+	100	+	100	+	70	(+)	100	+
ODC	0	-	0	-	0	-	0	-	0	-	0	-
LDC	0	-	0	-	3.2	-	0	-	0	-	0	-
Nitrate reduction	100	+	100	+	100	+	100	+	80	(+)	13.6	(-)
Indole production	4.5	-	100	+	28.4	(-)	100	+	50	v	0	-
α -haemolysis	18.2	(-)	0	-	1.1	-	0	-	0	-	45.5	v
Growth at:												
4°C	90.9	+	30.8	v	55.8	v	94.4	+	70	(+)	0	-
10°C	95.5	+	100	+	100	+	100	+	100	+	95	+
28°C	100	+	92.3	+	98.9	+	100	+	80	(+)	10	-
37°C	0	-	0	-	3.2	-	0	-	0	-	5	-
44°C	0	-	0	-	3.2	-	0	-	0	-	0	-
Growth in:												
0.5% NaCl	0	-	0	-	6.3	-	88.9	(+)	100	+	100	+
3.5% NaCl	100	+	100	+	27.4	(-)	100	+	100	+	100	+
5% NaCl	100	+	100	+	28.4	(-)	94.4	+	100	+	100	+
7% NaCl	68.2	v	38.5	v	35.8	v	38.9	v	0	-	0	-
10% NaCl	4.5	-	0	-	0	-	5.6	-	0	-	0	-
Crystal violet	0	-	0	-	2.1	-	16.7	(-)	10	-	68.2	v
TCBS Agar	100	+	100	+	98.9	+	100	+	100	+	100	+
TCBS (yellow)	50	v	76.9	(+)	74.7	(+)	88.9	(+)	100	+	100	+
Acid from:												
D-cellobiose	100	+	76.9	(+)	89.5	(+)	0	-	0	-	0	-
D-fructose	77.3	(+)	100	+	27.4	(-)	83.3	(+)	90	+	100	+
D-galactose	31.8	v	23.1	(-)	25.3	(-)	5.6	-	0	-	0	-
Glycerol	0	-	0	-	24.2	(-)	0	-	10	-	0	-
D-mannitol	81.8	(+)	76.9	(+)	86.3	(+)	0	-	0	-	0	-
D-mannose	54.5	v	100	+	88.4	(+)	11.1	(-)	10	-	9.1	-
Ribose	100	+	100	+	95.8	+	16.7	(-)	0	-	31.8	v
Sucrose	27.3	(-)	61.5	v	14.7	(-)	72.2	(+)	70	(+)	90.9	+
D-trehalose	50	v	92.3	+	91.6	+	66.7	v	100	+	86.4	(+)
Degradation of:												
Starch	95.5	+	100	+	100	+	72.2	(+)	100	+	100	+
Chitin	0	-	15.4	(-)	2.1	-	0	-	0	-	0	-
Gelatin	54.5	v	76.9	(+)	23.2	(-)	0	-	0	-	0	-
Casein	100	+	100	+	96.8	+	94.4	+	100	+	100	+
DNA	0	-	53.9	v	8.4	-	11.1	(-)	0	-	22.7	(-)
Lecithin	86.4	(+)	84.6	(+)	62.1	v	50	v	60	v	23.8	(-)
Chondroitin	50	v	76.9	(+)	2.1	-	5.6	-	0	-	0	-
Esculin	50	v	15.4	(-)	77.9	(+)	72.2	(+)	60	v	66.7	v
Agar	0	-	11.1	(-)	2.1	-	0	-	0	-	0	-
Use as sole carbon source:												
Acetate	90.0	+	0	-	0	-	0	-	0	-	4.6	-
β -alanine	4.5	-	0	-	0	-	0	-	0	-	4.6	-
DL-alanine	95.5	+	7.7	-	30.5	v	33.3	v	0	-	36.4	v
L-arginine	40.9	v	0	-	13.7	(-)	11.1	(-)	0	-	0	-
Citrate	100	+	7.7	-	82.1	(+)	22.2	(-)	0	-	45.5	v
Glycine	86.4	(+)	76.9	(+)	15.8	(-)	22.2	(-)	0	-	95.5	+
Inulin	0	-	0	-	1.1	-	0	-	0	-	0	-
L-lysine	4.5	-	7.7	-	0	-	0	-	0	-	0	-
Malonate	4.5	-	0	-	0	-	0	-	0	-	0	-
L-phenylalanine	0	-	0	-	0	-	0	-	0	-	0	-
L-proline	95.5	+	76.9	(+)	96.8	+	83.3	(+)	60	v	100	+
Propanol	18.2	(-)	0	-	1.1	-	0	-	0	-	0	-
Pyruvate	86.4	(+)	69.2	v	87.4	(+)	100	+	10	-	100	+
L-serine	90.9	+	61.5	v	65.3	v	83.3	(+)	10	-	45.5	v
Succinate	95.5	+	7.7	-	74.7	(+)	88.9	(+)	20	(-)	100	+
L-tartrate	4.5	-	0	-	0	-	0	-	0	-	0	-
L-tryptophan	0	-	0	-	1.1	-	5.6	-	0	-	0	-
Uracil	0	-	7.7	-	0	-	0	-	0	-	0	-

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TABLE 2.

continued

Test	Phenon 1 N = 22		Phenon 4 N = 13		Phenon 5 N = 95		Phenon 6 N = 18		Phenon 7 N = 10		Phenon 14 N = 22	
Sensitivity to:												
O/129 (150 µg)	61.9	v	46.2	v	81.1	(+)	94.4	+	100	+	100	+
Catalase	54.5	v	100	+	100	+	100	+	100	+	66.7	v
ADH	16.7	(-)	100	+	14.7	(-)	100	+	50	v	100	+
ODC	0	-	0	-	47.1	v	0	-	0	-	0	-
LDC	0	-	0	-	79.4	(+)	0	-	0	-	0	-
Nitrate reduction	100	+	100	+	100	+	100	+	50	v	100	+
Indole production	100	+	92.9	+	97.1	+	100	+	0	-	100	+
α-haemolysis	0	-	0	-	2.9	-	0	-	0	-	33.3	v
Growth at:												
4°C	0	-	100	+	17.7	(-)	0	-	0	-	0	-
10°C	ND	ND	100	+	10	-	100	+	50	v	66.7	v
28°C	100	+	100	+	10	-	100	+	100	+	100	+
37°C	0	-	7.1	-	100	+	33.3	v	50	v	0	-
44°C	0	-	0	-	91.2	+	33.3	v	50	v	0	-
Growth in:												
0.5% NaCl	0	-	100	+	100	+	100	+	50	v	100	+
3.5% NaCl	100	+	100	+	100	+	100	+	100	+	100	+
5% NaCl	100	+	100	+	100	+	100	+	100	+	100	+
7% NaCl	58.3	v	78.6	(+)	100	+	66.7	v	100	+	66.7	v
10% NaCl	0	-	14.3	(-)	76.5	(+)	0	-	100	+	66.7	v
Crystal violet	8.3	-	7.1	-	82.4	(+)	0	-	0	-	0	-
TCBS Agar	100	+	100	+	97.1	+	100	+	0	-	100	+
TCBS (yellow)	100	+	92.9	+	88.2	(+)	0	-	0	-	100	+
Acid from:												
D-cellobiose	100	+	0	-	0	-	0	-	0	-	0	-
D-fructose	ND	ND	42.9	v	94.1	+	100	+	0	-	100	+
D-galactose	50	v	0	-	0	-	0	-	0	-	0	-
Glycerol	25	(-)	42.9	v	23.5	(-)	0	-	0	-	0	-
D-mannitol	91.7	+	0	-	32.4	v	100	+	0	-	0	-
D-mannose	100	+	28.6	v	35.3	v	66.7	v	0	-	33.3	v
Ribose	100	+	35.7	v	61.8	v	100	+	0	-	0	-
Sucrose	100	+	92.9	+	79.4	(+)	0	-	0	-	100	+
D-trehalose	ND	ND	92.9	+	100	+	100	+	0	-	66.7	v
Degradation of:												
Starch	0	-	ND	ND	ND	ND	100	+	0	-	100	+
Chitin	0	-	7.1	-	0	-	66.7	v	0	-	33.3	v
Gelatin	8.3	-	0	-	2.9	-	33.3	v	0	-	0	-
Casein	0	-	100	+	79.4	(+)	100	+	ND	ND	0	-
DNA	0	-	0	-	35.3	v	0	-	ND	ND	0	-
Lecithin	0	-	ND	ND	ND	ND	66.7	v	ND	ND	33.3	v
Chondroitin	50	v	0	-	0	-	0	-	ND	ND	0	-
Esculin	100	+	0	-	44.1	v	0	-	ND	ND	33.3	v
Agar	0	-	0	-	0	-	0	-	0	-	33.3	v
Use as sole carbon source:												
Acetate	0	-	28.6	(-)	97.1	+	33.3	v	100	+	0	-
β-alanine	0	-	42.9	v	73.5	(+)	0	-	50	v	0	-
DL-alanine	ND	ND	85.7	(+)	100	+	66.7	v	100	+	0	-
L-arginine	ND	ND	14.3	(-)	88.2	(+)	100	+	100	+	0	-
Citrate	0	-	85.7	(+)	73.5	(+)	0	-	100	+	0	-
Glycine	66.7	v	64.3	v	29.4	(-)	100	+	50	v	0	-
Inulin	0	-	71.4	(+)	8.8	-	33.3	v	100	+	0	-
L-lysine	0	-	42.9	v	55.9	v	0	-	100	+	0	-
Malonate	0	-	64.3	v	76.5	(+)	0	-	50	v	0	-
L-phenylalanine	0	-	14.3	(-)	82.4	(+)	0	-	100	+	0	-
L-proline	83.3	(+)	100	+	29.4	(-)	100	+	100	+	0	-
Propanol	0	-	71.4	(+)	26.5	(-)	0	-	100	+	0	-
Pyruvate	58.3	v	100	+	29.4	(-)	100	+	100	+	100	+
L-serine	0	-	100	+	29.4	(-)	100	+	100	+	0	-
Succinate	0	-	100	+	29.4	(-)	100	+	100	+	0	-

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TABLE 2.

continued

Test	Phenon 1 N = 22		Phenon 4 N = 13		Phenon 5 N = 95		Phenon 6 N = 18		Phenon 7 N = 10		Phenon 14 N = 22	
L-tartrate	0	—	28.6	(—)	52.9	v	0	—	100	+	0	—
L-tryptophan	0	—	64.3	v	67.7	v	0	—	100	+	0	—
Uracil	41.7	v	50	v	67.7	v	0	—	50	v	0	—
Sensitivity to:												
O/129 (150 µg)	75	(+)	85.7	(+)	82.4	(+)	ND	ND	100	+	100	+

"N" indicates the number of isolates included in each phenon: 1.-*Vibrio splendidus* biotype I; 4.-*V. (Listonella) anguillarum*; 5.-*V. aestuarianus*; 6.-*V. anguillarum*; 7.-*V. mytili*; 14.-*V. mytili*; 17.-*V. splendidus* biotype I; 18.-*V. salmonicida*; 21.-*V. alginolyticus*; 22.-*V. furnissii*; 24.-*Photobacterium damsela* subsp. *piscicida*; 29.-*V. orientalis*.

Discriminatory tests for the biochemical identification of strains isolated from bivalve molluscs that are Gram negative, motile, oxidase positive, methyl red positive, facultative anaerobic and able to grow at 22 °C are expressed in bold.

Criteria (data are expressed in percentage of positive results): ND, No data; +, Positive result ($\geq 90\%$ of positive results); —, Negative result ($\leq 10\%$ of positive results); (+), Mainly positive results ($\geq 70\%$ < 90% of positive results); (—), Mainly negative results ($\geq 10\%$ < 30% of positive results); v, Variable results (>30% < 70% of positive results).

V. (Listonella) anguillarum

This group included phenon 4 (12 isolates and the reference strain of *V. (Listonella) anguillarum* NCIMB 571) and phenon 6 (17 isolates and the reference strain of *V. (Listonella) anguillarum* 43-F). These strains showed negative responses in tests for ornithine and lysine decarboxylase, growth in media containing crystal violet or 10% NaCl, growth at 37 °C and use of β -alanine, malonate, or propanol as sole carbon sources. They showed positive results in tests for catalase, nitrate reduction, indole production, Thornley's arginine dihydrolase, hydrolysis of starch or casein, and the use of L-proline as sole carbon source.

V. salmonicida

Phenon 18 (13 isolates and a reference strain of *V. salmonicida*). These strains were unable to carry out lysine or ornithine decarboxylation, growth in 10% NaCl or at 37 °C and hydrolysis of esculin or gelatin. They were able to grow at 4 °C and sensitive to O/129 (150 µg/disc).

V. furnissii

Phenon 22 (2 isolates and a reference strain of *V. furnissii*). These isolates had negative responses for the following tests: growth at 4 °C, in 10% NaCl or in crystal violet, ornithine or lysine decarboxylation, hydrolysis of chondroitin, acid production from D-cellobiose, and the use of malonate as sole carbon source. They showed positive results for Thornley's arginine dihydrolase test, nitrate reduction, catalase test, hydrolysis of starch or casein, acid production from D-mannitol or D-trehalose and use of L-arginine, L-proline, L-serine, or succinate as sole carbon source.

V. orientalis

Phenon 29 (2 isolates and a reference strain of *V. orientalis*). These strains showed negative results for growth at 37 °C and ornithine decarboxylation tests. However, they were able to grow at 28 °C; in 3.5% or 5% NaCl; showed positive results on indole production and nitrate reduction tests; were able to produce acid from D-fructose and sucrose; were sensitive to O/129 (150 µg/disc); and could use pyruvate as sole carbon source.

Ph. Damsela subsp. *Piscicida*

Phenon 24 (1 isolate and a reference strain of *Ph. damsela* subsp. *piscicida*). Both strains were unable to grow at 4 °C, on TCBS medium, carry out ornithine or lysine decarboxylation, indole production, hydrolysis of gelatin, acid production from D-cellobiose, glycerol, D-mannose, sucrose, or D-trehalose. Both strains showed positive results for the following tests: growth in 3.5% NaCl, catalase, and O/129 (150 µg/disc) susceptibility.

The following 12 tests (Table 2) are proposed as discriminatory and useful for the biochemical identification of Gram negative, motile, oxidase positive, methyl red positive, facultative anaerobic, and able to grow at 22 °C strains associated with the culture of Galician bivalve mollusks: Thornley's arginine dihydrolase, lysine decarboxylase (Moeller medium), indole production, growth in different NaCl concentrations (0.5% and 7% NaCl), nitrate reduction, growth at 4 °C, acid production from D-fructose, sucrose and D-trehalose, and the use of citrate and pyruvate as sole carbon source.

Bacterial distribution

Vibrio species were the predominant bacterial strains associated with the culture of bivalve mollusks (Table 3). Bacterial strains isolated from the seed stage in clams were identified as *V. aestuarianus* (facultative anaerobic isolates) or *Pseudoalteromonas* spp. (aerobic isolates). This may be due to the low number of bacterial strains isolated at this stage. The only aerobic strains isolated from the larval and reproductive stages in clams were identified as belonging to the genus *Shewanella*. The seed and reproductive stages of *Ostrea edulis*, the latter with the highest number of isolates, showed *Vibrio*, *Shewanella*, and *Pseudoalteromonas* species. A large variety of aerobic strains and different *Vibrio* species could be identified in the larval stage of *Ostrea edulis*. Species of *Vibrio*, *Photobacterium*, *Pseudoalteromonas*, and *Pseudomonas* were identified from water samples. The presence of the 2 main identified species (*V. aestuarianus* and *V. splendidus* biotype I) in water samples and associated with different phases in the culture of oysters demonstrates the influence of the microflora in the surrounding water. Aerobic strains with low-NaCl requirements for growth and able to tolerate concentrations of up to 7% (w/v) NaCl were isolated from clams, oysters, water,

TABLE 3.
Origin of identified and unidentified isolates.

Identification	Clam Oyster						Water	Phyto*
	Seed	Larva	Rep.*	Seed	Larva	Rep.*		
† <i>Vibrio aestuarianus</i>	+	—	—	+	+	+	+	—
† <i>Vibrio splendidus</i> biotype 1	—	—	+	—	+	+	+	—
† <i>Vibrio alginolyticus</i>	—	+	—	+	—	+	—	—
† <i>Vibrio mytili</i>	—	+	—	+	—	+	—	—
† <i>V. (Listonella) anguillarum</i>	—	—	—	—	+	+	—	—
† <i>Vibrio salmonicida</i>	—	+	—	+	—	+	—	—
† <i>Photobacterium phosphoreum</i> / <i>Plesiomonas shigelloides</i>	—	—	+	—	—	—	—	—
† <i>Vibrio furnissii</i>	—	—	—	—	—	+	—	—
† <i>Vibrio orientalis</i>	—	+	—	—	—	—	—	—
† <i>Ph. damsela</i> subsp. <i>piscicida</i>	—	—	—	—	—	—	+	—
†No. of unidentified isolates	3	13	23	6	19	75	11	—
‡ <i>Shewanella</i> spp.	—	+	+	+	+	+	—	—
‡ <i>Pseudoalteromonas</i> spp.	+	—	—	+	+	+	+	+
‡ <i>Pseudomonas</i> spp.	—	—	—	—	+	—	+	+
‡ <i>Alcaligenes denitrificans</i>	—	—	—	—	+	—	—	—
‡Low-NaCl requirements	+	—	—	—	+	+	+	+
‡No. of unidentified isolates	3	1	1	3	12	3	4	2
Total number of isolates	10	33	48	31	80	238	43	5

†, Facultative anaerobic strains. ‡, Aerobic strains. The "+" symbol denotes the presence of one species in a specific sample and the "—" symbol indicates its absence.

*, Rep. = Reproductive; Phyto., Phytoplankton.

and phytoplankton samples, revealing the influence of living in estuarine water.

Excluding samples isolated from phytoplankton, where facultative anaerobic strains were not detected, each remaining sample showed bacterial strains up to now unidentified.

DISCUSSION

A comparison between aerobic and facultative anaerobic strains reveals the existence of a wide diversity among the aerobic isolates. Although 352 facultative anaerobic isolates were clustered in 41 phena (see Fig. 2), and one already contained 94 of these isolates, only half of the aerobic strains could be clustered: 52 isolates in 23 phena (see Fig. 1). This shows a proportion of 8.59 facultative anaerobic strains per phenon, versus a ratio of 2.26 aerobic strains per phenon. For facultative anaerobic strains the isolates were very similar, unlike the aerobic isolates (Ortigosa et al. 1994b, Pujalte et al. 1999, Macián et al. 2000).

Shewanella, *Pseudoalteromonas* and *Pseudomonas* were the main aerobic bacteria groups and *Vibrio aestuarianus*, *V. splendidus* biotype 1, *V. alginolyticus*, *V. mytili*, and *V. (Listonella) anguillarum* were the predominant facultative anaerobic bacteria identified in this study as being associated with the culture of bivalve mollusks in Galicia. Other authors have identified these microorganisms from oysters, clams, and other bivalve mollusks with a different predominance of species: Ortigosa et al. (1994b) studied aerobic, gram negative bacteria associated with oysters and surrounding water in Valencia (Spain), identifying species of *Alteromonas*, *Shewanella*, *Deleya*, *Flavobacterium*, *Oceanospirillum*, *Pseudomonas*, and marine *Agrobacterium*-like organisms. Montilla et al. (1994) found that the most frequent *Vibrio* species associated with clams, oysters, and mussels in nurseries at the Ebro Delta (Spain) was *V. fluvialis* followed by *V. pelagius*, *V. tubiashii*, *V. splendidus*, *V. alginolyticus* and *V. parahaemolyticus*. Hariharan

et al. (1995) studied bacteria associated with oysters and mussels from six river systems in Prince Edward Island (Canada); they identified *V. (Listonella) anguillarum* as the most common species from oysters and *V. alginolyticus*, and *V. splendidus* as the most common from mussels, with the second largest group of bacteria being *Pseudomonas/Shewanella*. Pujalte et al. (1999) studied bacteria associated with the Spanish Mediterranean coast and found that *V. splendidus* and *V. harveyi* were the main bacteria associated with oysters, whilst *Halomonas*, *Alteromonas* and *Pseudomonas* were the principal groups identified from seawater. Mauger et al. (2000) studied brackish waters and mussels in the northeast of Sicily (Italy) and identified the following *Vibrio* species: *V. fluvialis*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, and *V. mimicus*. Castro et al. (2002) found that the main *Vibrio* species associated with cultured Manila clams from the Atlantic coast of south-western Spain were *V. tubiashii*, *V. splendidus* biotype 1, and *V. harveyi*. Our results are in agreement with all the above mentioned studies, which show a clear prevalence of different species of genus *Vibrio*, followed by a different dominance of aerobic strains. This demonstrates the considerable influence of the specific natural conditions of each region on the colonization of cultures of bivalve mollusks.

The characteristics described in this study for each identified group are consistent with results previously reported by other authors for facultative anaerobic (West et al. 1986, Pujalte et al. 1993, Alsina & Blanch 1994a, Cerdá-Cuéllar et al. 1997, Thyssen et al. 1998) and aerobic strains (Bowman 1998, Sawabe et al. 1998, Venkateswaran et al. 1999), whom groups establishment allowed a great identification but only up to a genus level. As shown in Tables 1 and 2, the results of this study make it possible to improve the characterization of *Vibrio* species and aerobic isolates. The set of tests selected for identifying the *Vibrio* species obtained from the cultures of bivalve mollusks in Galicia are simi-

lar to those previously reported by other authors (West et al. 1986; Pujalte et al. 1993; Alsina & Blanch 1994a, Cerdá-Cuéllar et al. 1997, Thyssen et al. 1998, Zorrilla et al. 1999).

We have identified a single isolate of *Ph. damsela* subsp. *piscicida*, a causative agent of pasteurellosis (Zorrilla et al. 1999), associated with water samples (see Table 3). Also, *V. (Listonella) anguillarum*, a pathogen for fish, shellfish, bivalve mollusks, and other marine animals (Farmer & Hickman Brenner 1992), was isolated from oyster larvae and reproductive oysters. Nevertheless, we could detect neither *V. tubiashii*, a pathogen of oysters and clams (Hada et al. 1984) nor *V. tapetis*, the causative agent of the brown ring disease in clams (Borrego et al. 1996). Although previously associated with diseased wild octopus in NW of Spain (Farto et al. 2003), in our study, undertaken in the same area, *V. lentus* could not be isolated from bivalve mollusks cultures. *V. alginolyticus*, a potential pathogen for humans and shellfish, was isolated from clam larvae and seeds and reproductive oysters. The pathogens *V. vulnificus* and *V. parahaemolyticus* were not detected, even though they are common inhabitants of estuarine and marine environments and are frequently isolated from seawater and seafood (Maugeri et al. 2000). This concurs with the results of studies undertaken on oysters from the Mediterranean Sea (Ortí-gosa et al. 1994a, Pujalte et al. 1999), but contrasts with the abundance of these species in oysters from other geographical areas, such as the United States (O'Neill et al. 1990, Hlady et al. 1993, Wright et al. 1996) or Japan (Hor et al. 1995). The human pathogens *V. fluvialis* and *V. metschnikovii* were not detected. However, 43% of aerobic strains and 41% of facultative anaerobic strains remain unidentified.

In phenon 37 (see Fig. 2), seven facultative anaerobic isolates, associated with the reproductive stage in clams, showed a different identification depending on the data matrix used: they were iden-

tified as *Plesiomonas shigelloides* using the matrix of Bryant et al. (1986), or as *Photobacterium phosphoreum* according to that of Alsina and Blanch (1994a). The probability level of being one or the other species was very high, 98%, for both species. It was not possible to differentiate between these two species with the 92 tests used in this study. More standard tests and molecular techniques should be included for a definite confirmation.

This is not an ecological study about variability through time, but the high number of strains we have isolated from bivalve mollusks cultures in different geographical areas during a long time period, as well as the method we have used to obtain them, gives these isolates a representative value of the most frequent culturable bacteria associated with the culture of bivalve mollusks in the NW of Spain.

Numerical taxonomy is a well-accepted method to search for differential phenotypic characteristics among close taxonomic strains. This allows a rapid presumptive identification that is very useful in diagnostics and sanitation control analyses.

All discriminatory tests are presented to provide a rapid presumptive guide for identifying environmental and the most frequent pathogenic bacterial isolates from cultures of Galician bivalve mollusks. This guide has proved to be very useful for differentiating the high bacterial variability that we have found in this study.

ACKNOWLEDGMENTS

This work was supported by grants PGIDIT00MAR2002PR and PGIDIT02RMA30102PR from the *Xunta de Galicia*. The authors thank J. Montes and CECT for the kind provision of bacterial isolates and reference strains respectively, and also E. Longo for providing the multipoint inoculator.

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SOME ASPECTS OF MODELLING SCALLOP (*PECTEN MAXIMUS*, L.) DREDGE EFFICIENCY AND SPECIAL REFERENCE TO DREDGES WITH DEPRESSOR PLATE (ENGLISH CHANNEL, FRANCE)

SPYROS FIFAS,¹* JOËL VIGNEAU² AND WILLIAM LART³

¹IFREMER, Centre de Brest, DRV/RH, B.P. 70, 29280 Plouzané, France; ²IFREMER, Station de Port-en-Bessin, DRV/RH, Av. Gal de Gaulle, 14520 Port en Bessin, France ³SEAFISH, Seafish House, St Andrew's Dock, Hull, HU3 4QE, United Kingdom

ABSTRACT To estimate the abundance of scallop populations either by VPA approach or by directed survey on seabeds it is necessary to evaluate the efficiency of fishing gears. The age-size structure of commercial or experimental catches does not usually correspond to that of the targeted population. Several previous studies on scallop stocks in Scottish or English Channel waters show that different functions can be fitted on relationships of dredge efficiency versus scallop size (monotonic increasing functions or curves including increasing and decreasing stages). The two ways to tackle the question involve to calculate efficiency estimates, absolute (by diving after dredge haul) or relative (by comparing two different parameter dredges in standardized sampling conditions). This study develops aspects of methodology applied on calculation of dredge efficiency parameters. The study focuses on data of English Channel French scallop fisheries.

KEY WORDS: scallop, dredge, depressor plate, efficiency, logistical curve, variance, Taylor's polynomial

INTRODUCTION

In the assessment of the abundance and biomass of scallop populations, there is a requirement to calculate the efficiency and selectivity of the fishing gear. An extrapolation has to be made because age-size composition of commercial or experimental catches does not correspond to that of the actual population. A multiplicative factor including tow components has to be taken into account: (1) the first component is a correction rate independent of scallop size because only a part of the individuals are retained by dredging and (2) the second factor depends on size because of selectivity component through meshes and tooth spacing (Dare et al. 1993, Fifas & Berthou 1999).

After several recommendations for general methodology, we report on an evaluation of dredge efficiency experiment either using divers (Saint Brieuc Bay, Western English Channel; Fig. 1) or using two different mesh size dredges (Bay of Seine; Eastern English Channel; see Fig 1). In both cases, the experimental fishing gears are dredges fitted with a depressor plate (Fig. 2) and equipped with a belly and back ring size of 50 mm.

In the first case, the estimator is called absolute efficiency written as (Laurec & Le Guen 1981, Buestel et al. 1985):

$$\text{absolute efficiency} = \frac{\text{number of scallops in the dredge}}{\text{total number of scallops (dredge + sea bottom)}} \quad (1)$$

In the second case, we usually compare two different mesh size dredges (Vigneau et al. 2001). The experiment can also be performed between two types of vessels; Weinberg et al. (2000) compared swept area estimates of research and commercial vessels on catches of Atlantic surfclam (*Spisula solidissima*). The comparative estimator called relative efficiency is expressed by:

$$\text{relative efficiency} = \frac{\text{number of scallops in the first dredge (or vessel)}}{\text{number of scallops in the second dredge (or vessel)}} \quad (2)$$

Components of Efficiency

Dickie (1955), Dupouy (1982), and Dare & Palmer (1994) calculated dredge efficiency by tagging/recapture of scallops. Chapman et al. (1977) and Mason et al. (1979) worked on standard and spring-loaded dredges by using divers to survey the track after dredging; the same approach was also developed on dredges with depressor plate by Buestel et al. (1985); Beukers-Stewart et al. (2001) estimated absolute and relative efficiency of spring-loaded dredges. Similar studies were conducted on other species (e.g. oysters: *Ostrea luaria*, Allen & Cranfield 1979; *Tiostrea chilensis*, Doonan et al. 1994; black scallop, *Chlamys varia*: Shafee 1979; blue crab, *Callinectes sapidus*: Zhang et al. 1993; Voelstad et al. 2000; blue mussel, *Mytilus edulis*: Dolmer et al. 1999). Fifas (1991) and Fifas and Berthou (1999) also adopted the diving method for European scallops and argued that dredge efficiency in the case of fishing gear equipped with depressor plate can often be fitted by increasing monotonic curves (logistical equation).

Components of dredge efficiency were listed by Dare et al. (1993) for spring-loaded gears and by Vigneau et al. (2001) for gears with depressor plate. This review is completed by Horton (personal communication):

- (1) Combination of vessel speed/warp length: combination of both parameters exert influence on dredge towing tension: to stabilize towing efficiency, the higher dredging speed the longer warp (e.g. warp length is usually equal to three times depth for a dredging speed of three knots).
- (2) Water depth: Water depth potentially introduces an auxiliary variable of efficiency related to warp weight (depending on warp diameter and material) even if warp length is optimized against vessel speed. Potentially an increase of water depth reduces dredge penetration in sediment because the force exerted on warp in traction is the resulting vector of towing traction and warp weight (warp buoyancy can be neglected).
- (3) Direction of tow in relation to tidal flow: experience of commercial skippers confirmed by diving operations during scientific surveys emphasizes the effect of tow direction against tide: it was conclusively proved that the direc-

*Corresponding author. E-Mail: Spyros.Fifas@ifremer.fr



Figure 1. The main scallop fisheries of the area studied: the Saint-Brieuc Bay and the Bay of Seine in the English Channel.

tion of tow has to be directed against the tidal current of weak or mean coefficient (e.g. less than 90 in the Western English Channel) to reduce random variations of dredge efficiency.

- (4) Compression of tooth bar spring (spring-loaded dredges): Fishermen modify the spring extension according to empirical observations of catch and dredge behavior. They try different spring tensions on each dredge on a given seabed type until dredges start to catch scallops and then adjust the springs of the other dredges to be the same as the one that is catching optimally. Skippers also use other clues to dredge functioning: vibrations through the warps, wear on the teeth and other parts of the dredge and quantity of stones caught. Compressed spring are considered more adequate on flat substrates (the tooth angle is constant and the fishing gear penetrates in the sediment) whereas extended one is more efficient on rougher seabed (the tooth angle is variable and the dredge is able to fish among rocks and sandbanks). However, the approach varies between skippers.
- (5) Inclination of pressure plate (dredges with depressor plate): The depressor plate has two roles: to increase the stability of the dredge on the seabed and potentially to facilitate entry of material in the dredge bag owing to deflection of water at the entrance to the dredge. Too inclined pressure plate may create water turbulence that obstructs entry of scallops in the bag (Baird 1957).
- (6) Tooth length and spacing: Baird (1957) underlined the role of dredge teeth not only on scallop selection, but also on by-catch quantities and substrate fragments. In Fifas and Berthou (1999), tooth length of an experimental dredge was varied according to the nature of the sediment: for soft sea bottom, tooth length is 130 mm, and, for rough sea bottom, the length is 90 mm. Shorter length and reduced spacing results in more rapid filling up of the dredge bag with catch and by-catch. Lart (2003) found that tooth spacing affected selectivity in spring toothed dredges.
- (7) Mesh back size and material: Dupouy (1982), found that dredge efficiency on large scallops increased with increased mesh size (a 9% increase for mesh size of 90 mm compared with of 72 mm). In addition to a selectivity pro-

cess, a decrease in mesh size has the potential to modify the efficiency component independent of scallop size because of a reduction of hydrodynamic flow through the meshes.

- (8) Sediment type: apart from influence of seabed topography (rocks reefs and flat sea bottom; see # 4 earlier), substrate nature (sand, mud, gravels, and rocks) determines dredge efficiency (Laurec & Le Guen 1981). In Saint-Brieuc Bay, Buestel et al. (1985) estimated that efficiency of dredge with depressor plate at almost 0.67 on a soft sea bottom whereas it was equal to 0.30 on a rough seabed.
- (9) Tow duration and hence distance covered: It can be inferred from numbers 6 and 7 in this list that tow duration has to be adapted to avoid overfilling the dredge. This would explain Shafee's (1979) observation of a reduction of efficiency with increased haul duration for black scallop beds.
- (10) Population density: Zhang et al. (1993) report that dredge efficiency seems to have declined exponentially as blue crab density increased.

Matrix of Components

Among components listed above, we can neglect each independent variable that can be explained by another one. For example: (1) A combination of direction of tow, water depth and warp weight allows to calculate towing tension on sea bottom against observations at the surface; (2) biologic experiments can always be conducted against the tidal flow and during periods that do not generally exceed mean level coefficients to minimize as much as possible random variations of efficiency estimates; (3) the influence of mesh material can be neglected because the use of metal rings for the whole dredge back is generalized for the main French scallop fisheries; (4) sampling of scallop beds are usually carried out on heavily exploited populations where densities are likely to be low and thus components related to population density need not be taken into account.

After those simplifications, a square correlation matrix between efficiency parameters can be proposed at the aim of stabilizing efficiency value when a parameter is modified. The example cited in this study concerns spring-loaded dredges (Table 1).

Experiments carried out on dredge efficiency aim to eliminate the maximum number of parameters (by modeling, ANOVA or bootstrapping) that can be expressed by each other; only parameters with low correlations are of significant interest for analysis to avoid collinearity artefacts.

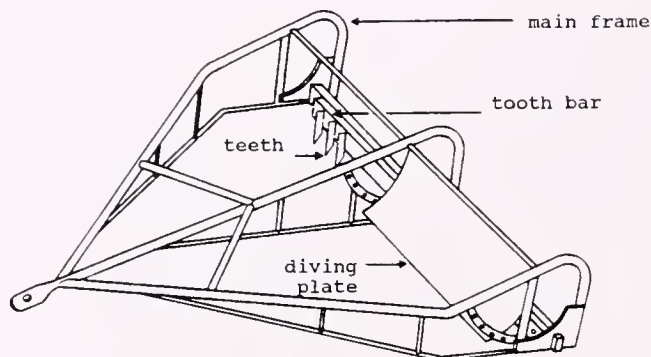


Figure 2. The experimental fishing gear. A dredge with a depressor (diving) plate.

TABLE I.

Square matrix of correlations between efficiency parameters. Example of spring-loaded dredges.

	Warp Tension	Spring Compression	Tooth Length	Tooth Spacing	Mesh Size	Sediment Type
Warp tension	1	0	???	0	0	0
Spring compression	0	1	???	???	???	F(X)
Tooth length	???	???	1	—	—	—
Tooth spacing	0	???	—	1	—	0
Mesh size	0	???	—	—	1	???
Sediment type	0	F(X)	—	0	???	1

0 = absence of correlation; 1 = collinearity; ??? = unknown (no experiment report); — = negative unknown value; F(X) = one of both parameters experimentally adjusted against the second one.

MATERIALS AND METHODS

Monotonic Functions for Dredges with Depressor Plate

The numerical examples reported in this study involve the analysis of the efficiency of dredges with depressor plate. In this particular dredge pattern, some simplifications are to be considered: compression does not exist while tooth angle and depth of teeth in sea bottom are reduced to only one independent variable (i.e. tooth length). An analysis of influence of tooth length is developed.

Previous studies (Buestel et al. 1985, Fifas 1991, Fifas & Berthou 1999, Vigneau et al. 2001) on scallop French fisheries (tooth length of 90–130 mm according to nature of the sea bottom) argued that experimental dredge efficiency, either absolute or relative, is explained by monotonic function versus scallop size (Fig. 3a and Fig. 3b). The same trends have been observed in the case of commercial dredges in Brittany (see Fig. 3c). Fifas and Berthou (1999) pointed out that efficiency is made up of the following coefficients:

- (1) A certain number of small sized scallops can escape through the 50 mm rings: this phenomenon defines the selectivity of dredge mesh related to escaping capacities of scallops that decrease as individual size increases.
- (2) Nevertheless, scallop dredges present a second type of selectivity, namely through the teeth that can be called the selectivity of the toothed bar. On the one hand, this phenomenon is similar to selectivity through the rings and can be described by an increasing monotonic function of the scallop size, but, on the other hand, it depends on the mechanical and physical conditions of dredging (e.g., the setting of the dredge due to the tooth length, meteorological conditions, etc.). This two-stage selection and retention process (by toothed bar and meshes) has already been described in the case of spring-loaded dredges (Dare et al. 1993 and Lart 2003).
- (3) A final component of efficiency is related to the mechanical and physical characteristics of dredging that are independent of scallop size. Even when individuals attain a large size and cannot escape through the teeth or the rings, all scallops are not caught on a dredged area: a certain number of them are still left on the bottom. This third term is defined as efficiency depending on the physical (nature of sea bottom, direction and speed of winds and currents, etc.) and mechanical (speed and direction of ship during the dredging, etc.) characteristics. If we consider that these

different parameters have a residual effect during stock assessment, we assume that, unlike the first two components of efficiency, the physical and mechanical components can be represented by a constant.

In regards to components as indicated earlier it is concluded that only one biologic component is included in usual efficiency models: this component is associated with escaping capacity of scallops and explains monotonic feature of efficiency against size.

In this case, absolute efficiency is represented by logistical functions (Fifas & Berthou 1999):

$$e_i = \frac{e_{\max}}{1 + \exp[-\alpha \cdot (L_i - L_{50})]} + \xi_i \quad (3)$$

with:

e_{\max} = maximum asymptotical efficiency; this term is considered to be independent of scallop size; it depends on physical and mechanical characteristics during dredging.

α = parameter linked to the deviation of selection of experimental dredge, defined by the difference $L_{75} - L_{25}$ of scallop sizes where efficiency is equal to 75% and 25%, respectively, of maximum asymptotical efficiency [$\alpha = 2 \cdot \ln(3) / (L_{75} - L_{25})$].

L_{50} = size corresponding to 50% of the maximum asymptotical efficiency.

ξ_i = unexplained residual error.

For a size L_i , the observed efficiency, e_i , is written as:

$$e_i = \frac{N_{i1}}{N_{i1} + N_{i2}} \quad (4)$$

where:

N_{i1} and N_{i2} = number of individuals of size L_i , caught by the dredge and those left on the sea bottom, respectively.

Furthermore, relative efficiency is a ratio of two logistical curves expressed by (Vigneau et al. 2001):

$$e_i = C \cdot \frac{1 + \exp[-\alpha' \cdot (L_i - L'_{50})]}{1 + \exp[-\alpha \cdot (L_i - L_{50})]} + \xi_i \quad (5)$$

where:

C = ratio of the two maximum asymptotical efficiencies ($C = e_{\max} / e'_{\max}$); the terms e_{\max} , e'_{\max} for the two mesh sizes (72 and 50 mm respectively) are considered according to assumptions above, see formula (3).

α and α' = parameters linked to the deviation of selection of experimental dredges for the two mesh sizes (72 and 50 mm respectively) defined as in equation (3).

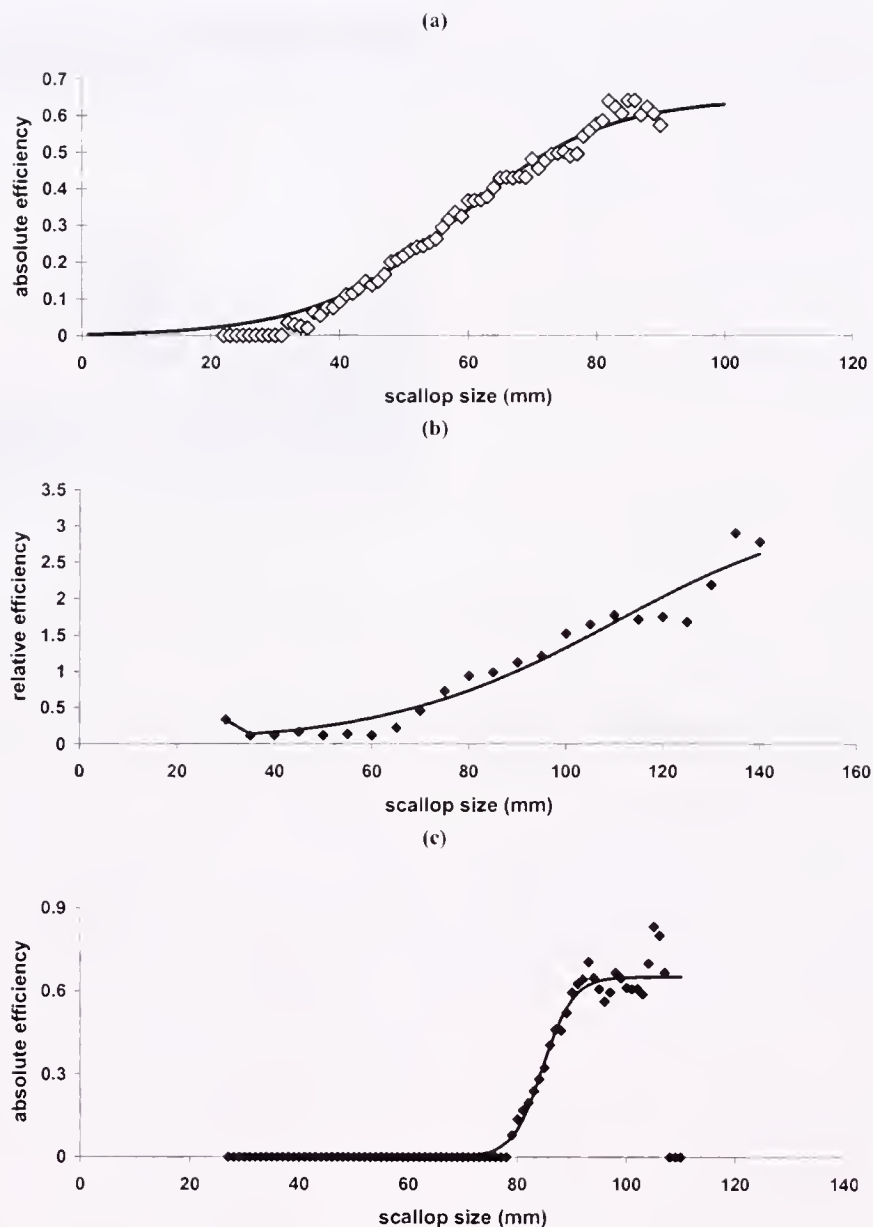


Figure 3. Previous studies on efficiency estimates of scallop dredges. (a) Absolute efficiency of experimental dredge in Saint-Brieuc Bay (mesh size of 50 mm; in Fifas and Berthon, 1999). (b) Relative efficiency of experimental dredges in Bay of Seine (mesh sizes of 50 and 85 mm; in Vigneau et al., 2001). (c) Absolute efficiency of commercial dredges in Brittany (mesh size of 92 mm; unpublished data; $e_{\max} = 0.652$; $\alpha = 0.363$; $L_{50} = 84.685$).

L_{50} and L'_{50} = size corresponding to 50% of the maximum asymptotical efficiency (for the mesh sizes of 72 and 50 mm).

For a size L_i , the observed efficiency, e_i , is expressed by:

$$e_i = \frac{N_i}{N'_i} \quad (6)$$

where:

N_i and N'_i = numbers of individuals of size L_i , caught by the two dredges (mesh sizes of 72 and 50 mm).

Composite Functions

The simplified modeling script of only one biologic component involving monotonic functions of efficiency is not always veri-

fied in experimental surveys. Sometimes, as described by Mason et al. (1979) on Scottish scallop fisheries exploited by spring-loaded dredges, a double model better describes efficiency versus size: one increasing function as logistic equations cited earlier, another one linear decreasing. Experimental data sampled during 1990s using dredges with depressor plate on scallop fisheries of the Western English Channel (tooth length of 75 mm) have revealed the same aspect: decreasing efficiency seems to start above a limiting size of 90–100 mm (see experimental data in Fig. 4). Only mathematical estimators of absolute efficiency will be presented later. A two-stage efficiency model can be expressed as:

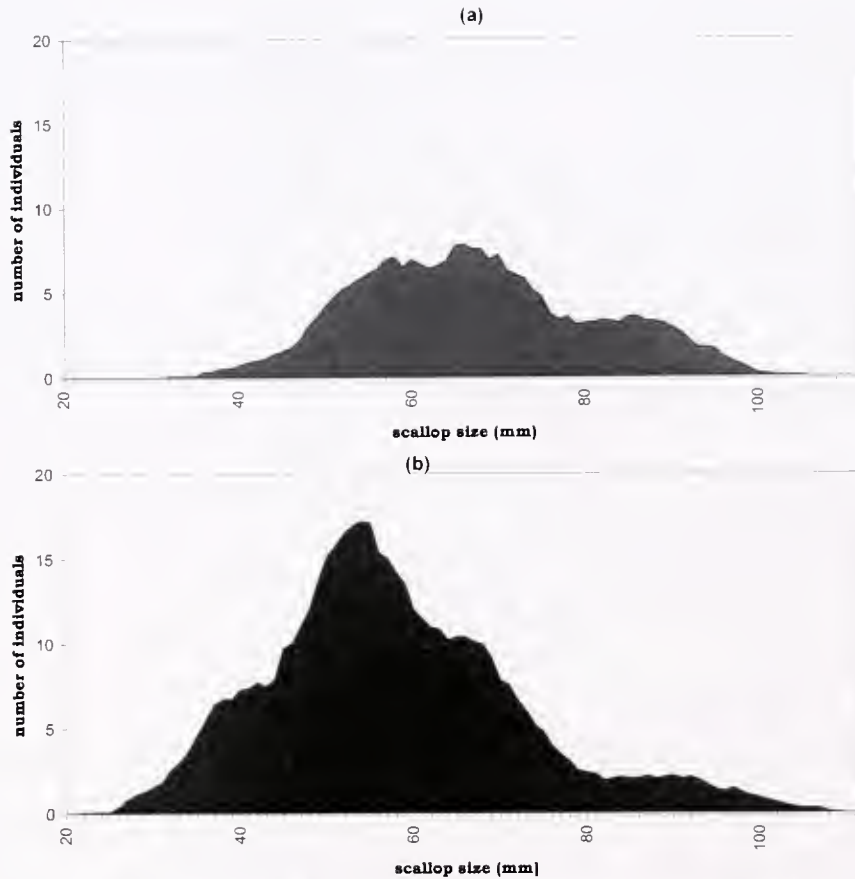


Figure 4. Distribution of size frequencies of scallops used in experiments: (a) Scallops caught by experimental dredges. (b) Escaping scallops harvested by diving on dredge track.

$$e_i = \frac{e_{\max}}{1 + \exp[-\alpha \cdot (L_i - L_{50})]} + \xi_i \quad \text{if } L_i \leq L_{\lim} \quad (\text{increasing logistical stage})$$

$$e_i = C \cdot \exp[-b \cdot L_i] + \xi_i \quad \text{if } L_i \geq L_{\lim} \quad (\text{decreasing exponential stage}) \quad (7)$$

Note: if b approaches zero, $C \cdot \exp[-b \cdot (L_i - L_{\lim})] \approx C \cdot [1 - b \cdot (L_i - L_{\lim})]$ and decreasing stage of efficiency against size is close to linear one described by Mason et al. (1979).

If the two stages of the model must give the same fitted value of efficiency for the limit size L_{\lim} , it is concluded that:

$$C = \frac{e_{\max} \cdot \exp[b \cdot L_{\lim}]}{1 + \exp[-\alpha \cdot (L_{\lim} - L_{50})]} \quad (8)$$

which reduces the number of unknown parameters of the efficiency model to five from six. Thus, the formula (7) can be written:

$$e_i = \frac{e_{\max}}{1 + \exp[-\alpha \cdot (L_i - L_{50})]} + \xi_i \quad \text{if } L_i \leq L_{\lim}$$

$$e_i = \frac{e_{\max} \cdot \exp[-b \cdot (L_i - L_{\lim})]}{1 + \exp[-\alpha \cdot (L_{\lim} - L_{50})]} + \xi_i \quad \text{if } L_i > L_{\lim} \quad (9)$$

For the fitting of the two-stage model different algorithms can be performed based on parametric approaches (e.g., conditional maximum likelihood model: Millar 1991; multinomial likelihood method: Perez-Comas & Skalski 1996) or on non-parametric ones

(e.g., Simplex method: Nelder & Mead 1965; complex method with constraint: Box, 1965). A comparison of direct and linear fitting methods was presented by Caddy and Defoe (1996). The fitting of the two-stage model has been conducted according to following principles:

- (1) The fitting has been done using Solver method of generalized reduced gradient, based on Newton's cancellation of the gradient (Waren & Lasdon 1979, Lasdon & Waren 1980, Lasdon & Waren 1983). Estimates of this parametric method were compared with those obtained by the Box (1965) non-parametric constrained estimation called Complex (including two constraints: $e_{\max} < 1$ and $L_{50} < L_{\lim}$).
- (2) The algorithm proceeds by minimizing the sum of the weighed residual squares: for a given size classe i , weight is equal to the corresponding total number of scallops, (i.e., scallops harvested by dredge) (N_{i1}), plus those sampled on the sea bottom by divers (N_{i2}). This procedure attributes greater weight to intermediate scallop size classes and probably reduces uncertainty in parameters α and L_{50} . The vector of parameters (e_{\max} , α , L_{50} , L_{\lim} , b) is estimated by minimizing the following quantity:

$$\sum_{i=1}^{nl} (N_{i1} + N_{i2}) \cdot \left[e_i - \frac{e_{\max}}{1 + \exp[-\alpha \cdot (L_i - L_{50})]} \right]^2 + \sum_{i=nl+1}^{nc} (N_{i1} + N_{i2}) \cdot \left[e_i - \frac{e_{\max} \cdot \exp[-b \cdot (L_i - L_{\lim})]}{1 + \exp[-\alpha \cdot (L_{\lim} - L_{50})]} \right]^2 \quad (10)$$

where: nc = total number of size classes taken into account for fitting; nl = number of size classes for $L_i \leq L_{lim}$.

Matrix of Variance-Covariance of Parameters

The Generalized Reduced Gradient and the Complex method do not give an estimate of the matrix of variances-covariances of the five parameters. In this case, it is usually recommended to apply non-parametric techniques such as the bootstrap method (Caddy & Defoe 1996). The calculation can also be carried out according to parametric procedure (Lin 1987, Fifas 1991) using Jacobian matrix (i.e., matrix of partial derivatives of the objective).

The matrix of variances-covariances is obtained by the following relationship:

$$[M] = s^2 \cdot [I]^{-1} \quad (11)$$

with:

$[M]$ = matrix of variances-covariances; $[I]^{-1}$ = inverse of matrix of information; s^2 = sum of mean residual squares of the fitted function:

$$s^2 = \frac{\sum_{i=1}^{nl} \left[e_i - \frac{e_{max}}{1 + \exp[-\alpha \cdot (L_i - L_{50})]} \right]^2 + \sum_{i=nl+1}^{nc} \left[e_i - \frac{e_{max} \cdot \exp[-b \cdot (L_i - L_{lim})]}{1 + \exp[-\alpha \cdot (L_{lim} - L_{50})]} \right]^2}{nc - 5} \quad (12)$$

The matrix of information is obtained by:

$$[I] = [J]' \cdot [J] \quad (13)$$

$[J]$ is the Jacobian matrix (nc rows and 5 columns):

$$[J] = \begin{bmatrix} \frac{\partial e_1}{\partial e_{max}} & \frac{\partial e_1}{\partial \alpha} & \frac{\partial e_1}{\partial L_{50}} & \frac{\partial e_1}{\partial L_{lim}} & \frac{\partial e_1}{\partial b} \\ \frac{\partial e_2}{\partial e_{max}} & \frac{\partial e_2}{\partial \alpha} & \frac{\partial e_2}{\partial L_{50}} & \frac{\partial e_2}{\partial L_{lim}} & \frac{\partial e_2}{\partial b} \\ \vdots & \vdots & \vdots & \vdots & \vdots \\ \frac{\partial e_{nc}}{\partial e_{max}} & \frac{\partial e_{nc}}{\partial \alpha} & \frac{\partial e_{nc}}{\partial L_{50}} & \frac{\partial e_{nc}}{\partial L_{lim}} & \frac{\partial e_{nc}}{\partial b} \end{bmatrix} \quad (14)$$

$[J]'$ is the transpose of $[J]$, the partial derivatives of the equation (14), also defined as absolute coefficients of sensitivity of order 1 written as $a(e_{max})$, $a(\alpha)$, $a(L_{50})$, $a(L_{lim})$ and $a(b)$, are presented in the annex 1 (formulae (18) to (22)) and the matrix of information $[I]$ is inverted using an algorithm presented by Lefebvre (1980).

The matrix of variances-covariances of the five parameters of the model and the use of partial derivatives of order 1 provide an approximate calculation of the variance of the variable $\Psi(L)$ corresponding to efficiency versus size L . This procedure is based on limited developments of order 1 in Taylor's series (called delta methods: Laurec 1986, Laurec & Mesnil 1987, Chevaillier 1990, Chevaillier & Laurec 1990, Fifas 1991).

By using Taylor's polynomial on a function Φ against parameters $\theta_1, \theta_2, \dots, \theta_k$ it is possible to present the variance of Φ by:

$$V[\Phi] \approx \sum_{i=1}^k \left(\frac{\partial \Phi}{\partial \theta_i} \right)^2 \cdot V[\theta_i] + 2 \cdot \sum_{i=1}^{k-1} \sum_{j=i+1}^k \frac{\partial \Phi}{\partial \theta_i} \cdot \frac{\partial \Phi}{\partial \theta_j} \cdot \text{Cov}[\theta_i, \theta_j] \quad (15)$$

In the case of the efficiency two-stage model, the formula (15) is equivalent to:

$$\begin{aligned} V[\Psi(L)] \approx & \left(\frac{\partial \Psi(L)}{\partial e_{max}} \right)^2 \cdot V[e_{max}] + \left(\frac{\partial \Psi(L)}{\partial \alpha} \right)^2 \cdot V[\alpha] \\ & + \left(\frac{\partial \Psi(L)}{\partial L_{50}} \right)^2 \cdot V[L_{50}] + \left(\frac{\partial \Psi(L)}{\partial L_{lim}} \right)^2 \cdot V[L_{lim}] \\ & + \left(\frac{\partial \Psi(L)}{\partial b} \right)^2 \cdot V[b] + 2 \cdot \frac{\partial \Psi(L)}{\partial e_{max}} \cdot \frac{\partial \Psi(L)}{\partial \alpha} \cdot \text{Cov}[e_{max}, \alpha] \\ & + 2 \cdot \frac{\partial \Psi(L)}{\partial e_{max}} \cdot \frac{\partial \Psi(L)}{\partial L_{50}} \cdot \text{Cov}[e_{max}, L_{50}] \\ & + 2 \cdot \frac{\partial \Psi(L)}{\partial \alpha} \cdot \frac{\partial \Psi(L)}{\partial L_{50}} \cdot \text{Cov}[\alpha, L_{50}] \\ & + 2 \cdot \frac{\partial \Psi(L)}{\partial e_{max}} \cdot \frac{\partial \Psi(L)}{\partial L_{lim}} \cdot \text{Cov}[e_{max}, L_{lim}] \\ & + 2 \cdot \frac{\partial \Psi(L)}{\partial e_{max}} \cdot \frac{\partial \Psi(L)}{\partial b} \cdot \text{Cov}[e_{max}, b] \\ & + 2 \cdot \frac{\partial \Psi(L)}{\partial \alpha} \cdot \frac{\partial \Psi(L)}{\partial L_{lim}} \cdot \text{Cov}[\alpha, L_{lim}] \\ & + 2 \cdot \frac{\partial \Psi(L)}{\partial \alpha} \cdot \frac{\partial \Psi(L)}{\partial b} \cdot \text{Cov}[\alpha, b] \\ & + 2 \cdot \frac{\partial \Psi(L)}{\partial L_{50}} \cdot \frac{\partial \Psi(L)}{\partial L_{lim}} \cdot \text{Cov}[L_{50}, L_{lim}] \\ & + 2 \cdot \frac{\partial \Psi(L)}{\partial L_{50}} \cdot \frac{\partial \Psi(L)}{\partial b} \cdot \text{Cov}[L_{50}, b] \\ & + 2 \cdot \frac{\partial \Psi(L)}{\partial L_{lim}} \cdot \frac{\partial \Psi(L)}{\partial b} \cdot \text{Cov}[L_{lim}, b] \end{aligned} \quad (16)$$

RESULTS

The results of fitting are presented in Table 2 that also provides the main parameters of the logistic curve fitted for increasing stage (deviation of selection, factor of selection) and the matrix of variances-covariances and of correlations between the parameters. The fitted efficiency model with mean confidence intervals (see calculation of variance in formula [16]) is given in Figure 5. Variance of efficiency calculated by using Taylor's polynomial is illustrated versus scallop size in Figure 6; absolute coefficients of sensitivity of order 1 for the 5 model parameters are given by Figure 7.

The estimated relationship of efficiency e_i against size L_i is written as:

$$\begin{aligned} e_i &= \frac{0.629}{1 + \exp[-0.095 \cdot (L_i - 57.824)]} & \text{if } L_i \leq 92.478 \\ e_i &= 0.607 \cdot \exp[-0.168 \cdot (L_i - 92.478)] & \text{if } L_i > 92.478 \end{aligned} \quad (17)$$

A general examination of the fitted function shows that the chosen two-stage model is well adopted. In fact, the maximum asymptotical value, e_{max} , of the increasing logistical stage equal to 0.629 is close to that of previous studies on the same area for large sized scallops: Buestel et al. (1985) estimated this value at 0.7. Furthermore, Fifas (1991) used a ratio estimator for scallops of age-group 3 and plus (including individuals larger than 80 to 90 mm) of 0.675 and Fifas and Berthou (1999) calculated a maximum asymptotical efficiency of 0.646. Thus, it is indicated indirectly that asymptotical efficiency level is independent of depth of toothed bar in the ground because all experiments cited earlier were undertaken with

TABLE 2.
Fitting of the two-stage function of efficiency.

Parameter	Value	Standard Deviation	C.V. (%)
e_{\max}	0.629	0.026	4.192
α (mm ⁻¹)	0.095	0.009	9.754
L_{50} (mm)	57.824	1.431	2.475
L_{\lim} (mm)	92.478	0.089	0.096
b (mm ⁻¹)	0.168	0.022	13.318

factor of selection: $L_{50}/L_{\text{mesh}}(50) = 1.156$
deviation of selection: $2.\ln(3)/\alpha = 23.015$ mm

	e_{\max}	α	L_{50}	L_{\lim}	b
Matrix of Variances-Covariances					
e_{\max}	0.696E-03	-0.188E-03	0.326E-01	-0.764E-03	0.000
α		0.867E-04	-0.880E-02	0.162E-03	0.000
L_{50}			0.205E+01	-0.340E-01	0.000
L_{\lim}				0.787E-02	0.140E-02
b					0.502E-03

	e_{\max}	α	L_{50}	L_{\lim}	b
Matrix of correlations					
e_{\max}	1.000	-0.764	0.863	-0.326	0.000
α		1.000	-0.661	0.196	0.000
L_{50}			1.000	-0.268	0.000
L_{\lim}				1.000	0.706
b					1.000

Number of size classes $nc = 83$, sum of weighed residual squares $SRQ = 1.474$.

various tooth length. In regards to unpublished fitted model for commercial dredge efficiency (see Fig. 3c), e_{\max} is also independent of mesh size (commercial dredges are equipped with 92 mm size, and biologic experiments are led with 50 mm meshes whereas both asymptotical levels are close); on this point, it is still difficult to conclude because observations on commercial efficiency have been carried out on only one vessel and fishing fleet variability is neglected.

In the same, comparison of the other parameters associated with the increasing stage of the model (α and L_{50}) reveals weak differences from equivalent values of monotonic functions (0.095 and 57.824 against 0.088 and 58.620, respectively, proposed by Fifas & Berthou [1999]) when only experimental dredge mesh size (50 mm) is considered. The most significant difference from pre-

vious results consists on existence of a decreasing stage similar to Mason et al. (1979) even if those authors proposed a linear model for the second stage. It is necessary to determine which is the design characteristic inducing the absence of asymptotical level on the efficiency curve. Most of the equipment of experimental dredges used by Fifas and Berthou (1999) and by this study were identical (dredge width, mesh size and material, tooth spacing, and inclination of the depressor plate); both dredges were also towed by the same vessel in similar meteorologic and tidal conditions. The difference in design designs consisted of differences in tooth length: 75 mm in this work against 90–130 mm used by Fifas and Berthou (1999) according to nature of the sea bed (soft or rough).

DISCUSSION

Physical and Biological Interpretation

In accordance with Baird (1957), use of short teeth on dredge bar modifies scallop selection and also quantity of non target spe-

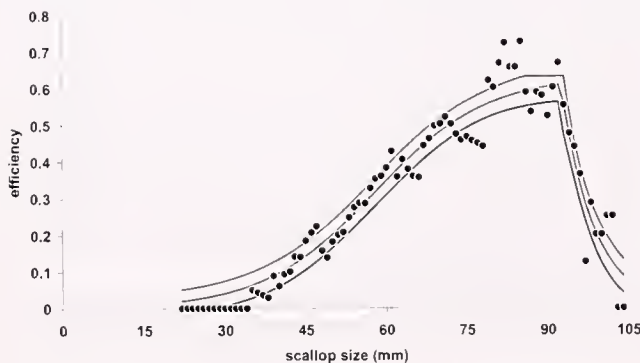


Figure 5. The fitted two-stage efficiency model with confidence intervals (confidence level $1-\alpha = 0.95$).

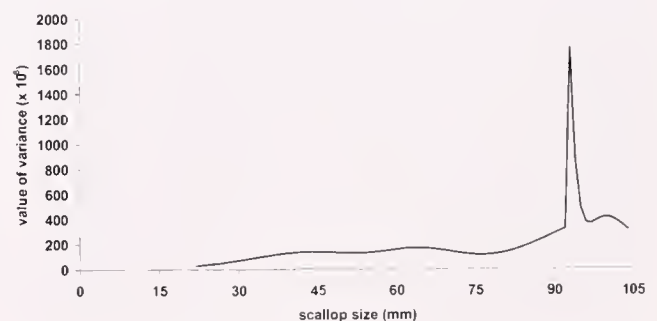


Figure 6. Variance of the two-stage efficiency against scallop size.

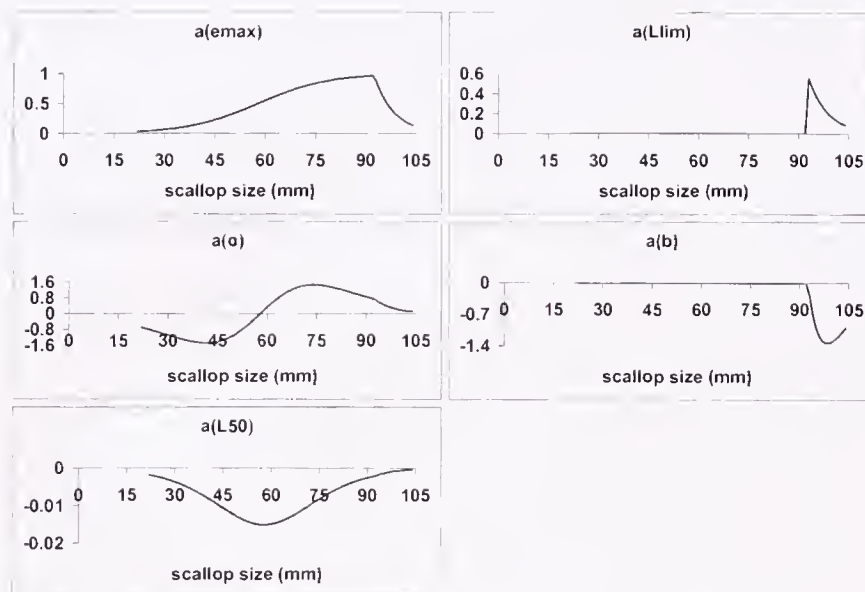


Figure 7. Absolute coefficients of sensitivity of order 1 of the 5 model parameters.

cies and substrate fragments. Other works discuss a combination of engineering and biologic approach on dredge design and gear efficiency (Cover & Sterling 1994 and Gaspar et al. 1999). A less penetration in the sediment, due to tooth length, causes a quicker filling up of the dredge bag. When filling up level is reached, hydrodynamic forces exerted through the meshes are significantly reduced and filtering capacities of dredge are minimized. A cylindrical strip of sediment is formed at the front of the dredge and obstructs entry of solid material in the bag. Hence, this implies decrease of forces leading scallops and by-catches into the dredge bag.

Thereby, beside the three components of efficiency models described by Fifas and Berthou (1999) (i.e., selectivity of bag meshes, selectivity of the toothed bar, maximum asymptotic level related to mechanical and physical characteristics of dredging), a fourth one (depletion of dredge efficiency after filling up in the case of short tooth length) has to be taken into account in agreement with Mason et al. (1979). Breaking of efficiency increasing stage seems to occur suddenly at a given scallop size (i.e., at a given weight: reduced hydrodynamic forces through meshes are probably not enough to attract heavier material (e.g., for 75 mm of tooth length, limit scallop size is equal to 92 mm i.e., almost 140 g of individual weight). Decreasing second stage of the curve against scallop size can be interpreted by 3 factors:

(1) Physical factor, resulting force from antagonism between scallop weight (increasing vs. size) and attractive component inward dredge bag (decreasing as and when filling up) is reduced; (2) Passive behavior factor, if dredge teeth are short, large scallops are probably more able to avoid dredges passively because of deeper digging in the sediment (i.e., masking their upper valve with sand). Sakurai and Seto (2000) found that behavior of Japanese scallops *Patinopecten yessoensis* depends on size while recessing by coating themselves with sand in depression; (3) active behavior factor, Scallop swim by clapping valves and water ejected from the mantle cavity during closing of the valves generates thrust that propels the animal. Many authors (Moore & Trueman 1971, Gruffydd 1976, Chapman 1981, Joll 1989, Dadswell & Weihs 1990, DeMont 1990, Arsenault & Himmelman

1996, Cheng & DeMont 1996, Fleury et al. 1996, Arsenault et al. 1997, and Sakurai & Seto 2000) debated on size-related swimming pattern of various pectinid species. In spite of differences between locomotion abilities of those species (e.g., *Amusium balloti* or *Chlamys islandica* do longer displacements than *Pecten maximus*), a common point emerges: small individuals swim more often, but effectiveness of movements caused by environmental disturbance grows with size.

Statistical Consequences of the Two-stage Model

The analysis of absolute coefficients of sensitivity for the efficiency one-stage model proposed by Fifas and Berthou (1999) was carried on three parameters, e_{\max} , α and L_{50} . These coefficients approach 0 asymptotically apart from the parameter e_{\max} close to 1 when scallop size L approaches $+\infty$. In the case of the two-stage model of this work, all sensitivity coefficients approach 0 because e_{\max} does not have the same sense: it is just a theoretical maximum value of the dredge efficiency for a narrow size interval near L_{\lim} . This is the main disadvantage due to the model discontinuity around L_{\lim} .

The coefficient $a(e_{\max})$ is represented by a curve close to fitted function. This fact indicates that for a given absolute error of e_{\max} , the error of the dependent variable (efficiency) increases for sizes below L_{\lim} , decreases for upper values.

The coefficients $a(\alpha)$ and $a(L_{50})$ are given by functions close to those investigated by Fifas and Berthou (1999), a non-symmetrical periodic function for the first one, a symmetrical at around L_{50} for the second. For the intermediate size range, the absolute errors of α or L_{50} contribute less significantly to the efficiency error. In the particular case of $L = L_{50}$, absolute error of α has a null contribution whereas negative effect of an absolute error of L_{50} is maximized.

The coefficient $a(L_{\lim})$ is maximized for $L = L_{\lim}$, then it declines exponentially. The maximum value is approximated by:

$$\frac{e_{\max}}{1 + \exp(-\alpha(L_{\lim} - L_{50}))} \quad (18)$$

(if we consider that α can be neglected comparatively to 1; see equation [21b]). In the scallop fishery of the Saint-Brieuc Bay, the contribution of an absolute error of L_{lim} is maximized for the frequently represented size classes around L_{lim} (90–100 mm).

The coefficient a (b) reaches its highest level for $L = L_{lim} + 1/b$ which is located in the same interval than above (90–100 mm), after that it converges slowly. As for L_{50} , the effect of an error of b is always negative.

Combined values of all those sensitivity coefficients with calculated variance-covariance parameters give a function of variance that does not have mathematically simple analytic formulation. It is characterized by 3 stages: (1) slow monotonical increase below L_{lim} (contribution of error of e_{max} grows to the detriment of α and L_{50}); (2) a very high peak for $L = L_{lim}$ (part of error of L_{lim} minimizes contribution of other four parameters) and (3) a decreasing stage for upper sizes (dominance of error of b). This investigation shows that efficiency two-stage model is well adapted for undersized individuals, but its use for great classes is disadvantaged. On the one hand, very high value of variance for L_{lim} requires a high accuracy on estimate of this parameter, but its physical interpretation is not easy (see earlier); on the other hand, contribution of b for large scallops indicates low precision of dredge efficiency because this parameter is the least accurate (see Table 2).

CONCLUSION

The fitting of the efficiency model is statistically satisfactory because it gives parameter coefficients of variation less than 10% to 15%. The data have been harvested over several years and this fact reduces the effects of inter-annual variability of efficiency, but it is necessary to verify if seasonal variability can be neglected because data were sampled during the same season. It would also be important to complete study by application of re-sampling techniques (bootstrap and jackknife). These methods cannot be developed on the data of this work because of the lack of information about the matching of harvested scallops to a given sample. The examination of absolute sensitivities and the analysis of the function of variance show the dominant role of the two parameters associated with the decreasing stage of the model. This point disadvantages efficiency models that are fitted on data collected by short toothed dredges because of reduction of accuracy of estimates for large scallop sizes. To assess scallop stocks by experimental surveys and to propose annual TAC, it is preferable to privilege stability of accuracy for as many scallop sizes as possible. Thus, it is better to use experimental dredges equipped by long tooth bars adapted according to nature of sea bottom. This

solution allows to calculate dredge efficiency characterized by relatively stable variance for exploited classes.

ANNEX 1

Partial derivatives of order 1 (absolute coefficients of sensitivity of order 1).

1. Parameter e_{max} :

$$a(e_{max}) = \frac{\partial e_i}{\partial e_{max}} = \frac{1}{1 + \exp[-\alpha \cdot (L_i - L_{50})]} \quad \text{if } L_i \leq L_{lim} \quad (19a)$$

$$a(e_{max}) = \frac{\partial e_i}{\partial e_{max}} = \frac{\exp[-b \cdot (L_i - L_{lim})]}{1 + \exp[-\alpha \cdot (L_{lim} - L_{50})]} \quad \text{if } L_i > L_{lim} \quad (19b)$$

2. Parameter α :

$$a(\alpha) = \frac{\partial e_i}{\partial \alpha} = \frac{e_{max} \cdot (L_i - L_{50}) \cdot \exp[\alpha \cdot (L_i - L_{50})]}{(1 + \exp[-\alpha \cdot (L_i - L_{50})])^2} \quad \text{if } L_i \leq L_{lim} \quad (20a)$$

$$a(\alpha) = \frac{\partial e_i}{\partial \alpha} = \frac{e_{max} \cdot (L_{lim} - L_{50}) \cdot \exp[-\alpha \cdot (L_{lim} - L_{50})] \cdot \exp[-b \cdot (L_i - L_{lim})]}{(1 + \exp[-\alpha \cdot (L_{lim} - L_{50})])^2} \quad \text{if } L_i > L_{lim} \quad (20b)$$

3. Parameter L_{50} :

$$a(L_{50}) = \frac{\partial e_i}{\partial L_{50}} = - \frac{\alpha \cdot e_{max} \cdot \exp[-\alpha \cdot (L_i - L_{50})]}{(1 + \exp[-\alpha \cdot (L_i - L_{50})])^2} \quad \text{if } L_i \leq L_{lim} \quad (21a)$$

$$a(L_{50}) = \frac{\partial e_i}{\partial L_{50}} = - \frac{\alpha \cdot e_{max} \cdot \exp[-\alpha \cdot (L_{lim} - L_{50})] \cdot \exp[-b \cdot (L_i - L_{lim})]}{(1 + \exp[-\alpha \cdot (L_{lim} - L_{50})])^2} \quad \text{if } L_i > L_{lim} \quad (21b)$$

4. Parameter L_{lim} :

$$a(L_{lim}) = \frac{\partial e_i}{\partial L_{lim}} = 0 \quad \text{if } L_i \leq L_{lim} \quad (22a)$$

$$a(L_{lim}) = \frac{\partial e_i}{\partial L_{lim}} = \frac{e_{max} \cdot \exp[-b \cdot (L_i - L_{lim})]}{1 + \exp[-\alpha \cdot (L_{lim} - L_{50})]} \cdot \frac{\alpha \cdot \exp[-\alpha \cdot (L_{lim} - L_{50})]}{1 + \exp[-\alpha \cdot (L_{lim} - L_{50})]} \quad \text{if } L_i > L_{lim} \quad (22b)$$

5. Parameter b :

$$a(b) = \frac{\partial e_i}{\partial b} = 0 \quad \text{if } L_i \leq L_{lim} \quad (23a)$$

$$a(b) = \frac{\partial e_i}{\partial b} = \frac{e_{max} \cdot (L_i - L_{lim}) \cdot \exp[-b \cdot (L_i - L_{lim})]}{1 + \exp[-\alpha \cdot (L_{lim} - L_{50})]} \quad \text{if } L_i > L_{lim} \quad (23b)$$

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ENRICHMENT OF TETRANUCLEOTIDE MICROSATELLITE LOCI FROM INVERTEBRATE SPECIES

K. H. KAUKINEN,* K. J. SUPERNALUT AND K. M. MILLER

Department of Fisheries and Oceans Canada, Science Branch, Pacific Biological Station,
Nanaimo, B.C., Canada V9R 5K6

ABSTRACT An experimental procedure using biotin labelled probes and streptavidin bound magnetic beads was developed to produce microsatellite-enriched libraries in two bivalve species, geoduck and Japanese scallop, and one crustacean, Dungeness crab. Microsatellite libraries were enriched for GATA₃ and GACA₄ by 75–84%. The magnetic bead protocol produced genomic inserts ranging in size from 347–449 bp, with an average 5' and 3' flanking of 89–130 bp. Eight polymorphic loci were isolated from both geoduck clam and Dungeness crab. The heterozygosities ranged from 0.57–0.92 for Dungeness crab and 0.91–0.97 for geoduck. These novel polymorphic microsatellite loci will be useful in studying recruitment dynamics and genetic structure.

KEY WORDS: microsatellites, enrichment, magnetic beads, geoduck, crab, scallop

INTRODUCTION

Marine invertebrate species with pelagic larval stages should have tremendous capacity for dispersal and mixing over long distances. However, numerous studies have shown that over ecological time scales (10's of years), dispersal in many species is primarily local (Swearer et al. 2002). Even on evolutionary time scales (100's to millions of years), while the majority of marine invertebrate species with pelagic larvae do not contain a high degree of spatial structure at the 10–100+ km scale, there are some that do (e.g. Jiang et al. 1995; Burton 1998). Further, many studies have noted fine-scale heterogeneity among cohorts (termed chaotic genetic patchiness in Hellberg et al. 2002) that suggests (1) that few adults contribute to each recruitment event (Hedgecock 1994), or (2) that there is selection on genetic diversity in the larval stage (Johnson and Black 1984). Because effective management units must incorporate information on recruitment dynamics and genetic structure, it is imperative that these parameters are understood for species of economic importance and those of conservation concern.

For genetic analyses of population structure, numerous genetic markers have been utilised, including allozymes, mtDNA, minisatellites, microsatellites, and other coding and non-coding loci. Microsatellite loci, which are simple sequence repeats with 2 to 10 bp motifs organised in tandem arrays, are currently considered the marker of choice for determining the level of population connectedness over the span of 100's to 1000's of years. Microsatellite loci are often highly polymorphic due to a high rate of mutation through replication slippage, resulting in the gain or loss repeat units. In addition, microsatellite loci are as vulnerable to point mutations as the rest of the genome, which tends to divide longer repeat stretches into smaller units, and hence decrease the rate at which slippage occurs (Bell and Jurka 1997; Kruglyak et al. 1998). This "slippage/point-mutation" theory suggests that the frequency distribution of microsatellite lengths is a balance of expansion due to slippage and contraction due to point mutation (Sibly et al. 2003).

To date, all prokaryotic and eukaryotic genomes have been found to contain microsatellite loci. However, among eukaryote species, microsatellite repeats are more abundant and longer in vertebrates than invertebrates (Chambers and MacAvoy 2000). As

a result, many researchers have experienced difficulty in isolating microsatellite loci from marine invertebrate species, especially tetranucleotide repeat loci which are generally preferred for population studies due to their lack of stutter. In a study comparing the prevalence of di-, tri-, tetra- and hexanucleotide repeats in shrimp, Xu et al. (1999) found that only 9% of microsatellite loci were in the form of tetranucleotide repeats. Similarly, using traditional library screening methods, we probed red sea urchin (*Strongylocentrotus franciscanus*) libraries with ³²P-labelled (GACA) and (GATA) oligonucleotides, and less than 10% of the microsatellite sequences obtained were tetranucleotide repeats (Miller et al.). Moreover, most of the tetranucleotide containing loci were not sufficiently polymorphic for use in population studies or pedigree analyses.

Numerous methods advancing the techniques used in the isolation of microsatellites have been developed. The first microsatellite enrichment protocol was described by Ostrander et al. (1992) and later expanded by Paetkau (1999). Under their protocol, the fractionated DNA was packaged into a phagemid or phage vector and an ssDNA library was obtained. The ssDNA was used as a template for PCR using the repeated oligonucleotides as the primers, thus creating double stranded product enriched for repeats. Both methods were reviewed in Zane et al. (2002), but it was noted that only five primer papers to date have been accredited to these approaches.

Fischer and Bachmann (1998) and Hamilton et al. (1999) described magnetic bead methods of microsatellite enrichment. Both groups used a linker ligated to the restricted DNA fragments as a primer for PCR reactions. The adapter-linked DNA was then used as the target for 5' biotinylated repeat oligonucleotides and paramagnetic streptavidin beads. Both groups used genomic:oligonucleotide hybrids as the target for streptavidin magnetic beads. Fischer and Bachmann reported a greater than 60% enrichment of microsatellites in the onion plant *Allium cepa*, while Hamilton and colleagues reported a 20–95% enrichment rate. In their review, Zane et al. (2002) presented their own magnetic approach to microsatellite isolation termed FIASCO (Fast Isolation by AFLP of sequences containing repeats). In principle, the DNA was digested simultaneously with the AFLP adaptor to achieve a one-step digestion-ligation reaction. As above, the adapter linked DNA was used in a PCR step, hybridised with biotin-(AC)₁₇ and separated with streptavidin beads resulting in a 50–95% enrichment for dinucleotide repeat microsatellites.

*Corresponding author. E-mail: Kaukinenk@df-mpo.gc.ca

The present study outlines the experimental procedure we developed to produce microsatellite-enriched libraries for marine invertebrate species. The magnetic bead enrichment approaches outlined above and in O'Reilly et al. (2000) form the basis of the methods developed, but substantial improvements were made to enhance the length of the core repeat and flanking sequence and the level of enrichment obtained. This procedure was applied in the development of highly polymorphic microsatellite loci from two gastropod species, geoduck (*Panopea abrupta*) and Japanese scallop (*Patinoplectin yessoensis*), and one crustacean species, Dungeness crab (*Cancer magister*). Primers to microsatellite loci for geoduck clams and Dungeness crab are described herein.

METHODS

Microsatellite enriched libraries were produced using magnetic bead hybridisation selection (Fig. 1). Genomic DNA was extracted from geoduck mantle, crab muscle, and scallop adductor muscle using a Stratagene DNA Extraction Kit (Stratagene, La Jolla, CA). Genomic DNA (approximately 50 µg) was partially digested with 10.8 U *Hae*III for 10, 20, and 30 min at 37°C. DNA fragments of 600–2000 bp were size selected and dephosphorylated by incubating 53 µl clean cut DNA with 10 U of CIP for 2 hr at 37°C. The CIP treated DNA was cleaned using spin columns (QIAquick PCR

purification kit, Qiagen, Valencia, CA) and eluted to a final volume of 60 µl in elution buffer.

The SNX linkers were designed according to Hamilton et al., 1999. Primers included SNX forward: 5'CTAAGGCCT-TGCTAGCAGAAGC3', and SNX reverse: 5'pGCTTCTGCTAG-CAAGGCCTTAGAAAA3'. The SNX linkers were added by PCR cycling in the presence of the SNX linkers, ligase and *Xmn*I. The ligation mixture contained 10 µl of CIP-treated DNA fragments, 3.9 mM mixed SNX reverse and forward, 1× Ligase Buffer, 1 U *Xmn*I and 2 U Ligase in a final volume of 30 µl. Ligations were cycled for 30' at 16°C, 10' at 37°C for 5–18 cycles, followed by 20' at 65°C to inactivate the enzymes, and then kept at 4°C. Different ligation cycling regimes were tested, and the optimum number of ligation cycles was empirically determined to be five. More than five cycles resulted in shorter fragments with multiple SNX linkers. The SNX ligation product was cleaned using spin columns and eluted to a final volume of 20 µl in EB.

The SNX PCR cocktail contained 0.8 mM SNX f, 0.8 mM dNTPs, 5 U Qiagen Taq, 2.5 mM MgCl₂, 10 µl SNX ligated DNA fragments, and 1× Qiagen PCR Buffer. The cycling protocol was 2' at 92°C followed by 40 cycles of 94°C/45", 62°C/1', 72°C/1', a final extension of 30'/72°C, and then held at 4°C. At this point, the PCR product can be immediately processed to the hybridisation step, size selected and concentrated, or cleaned with spin columns and retained for long-term storage. The ligation step can be tested by resolution on an agarose gel. A smear of products indicates a successful ligation and PCR.

All hybridisation steps were performed at 48°C in a controlled environment. All trays, tips, wash solutions and MPC (Molecular Particle Concentrator) were preheated to 48°C. Dynabead M-280 Streptavidin magnetic beads (DynaL ASA, Oslo, Norway) were washed 3 times with phosphate buffered saline with 0.1% sodium dodecyl sulphate using the Dynal MPC-S or -P (single or plate format) between washes. The beads were re-suspended in 5× SSC (20× SSC stock: 175.3g sodium chloride and 88.2g sodium citrate per litre; adjust pH to 7.0 with 10N hydrochloric acid). Previously, Hamilton et al. (1999) used genomic oligonucleotide hybrids as the target for streptavidin magnetic beads and O'Reilly et al. (2000) combined (GATA)₇ oligonucleotides bound to streptavidin coated paramagnetic beads with genomic DNA. We found that SNX-ligated DNA was efficiently enriched by shorter biotin-(GACA)₄ and (GATA)₄ oligonucleotides bound to streptavidin beads. Notably, 100 µl of magnetic beads were incubated with 1 µl (200 pmol) biotinylated oligo probes for 15 min at room temperature. The 5' biotinylated oligonucleotide primers were obtained from commercial sources (University of Calgary, AB, Canada). The bead:oligo complex was washed 3 times with 5× SSC using the MPC between washes, resuspended in 35 µl of 10× SSC, and held at 48°C for the hybridization steps. The target DNA was denatured by incubating 10 µl of SNX PCR product, 10 µl SNXf (competitor) (0.5 ng/µl) and 55 µl water at 95°C/15', then plunged into ice. The bead:oligo complex was combined with the denatured target (SNX PCR product+competitor) and incubated at 48°C for 60 min.

Some groups have reported successful enrichments at room temperature (RT) using magnetic beads (see Zane et al. 2002 and Fischer and Bachmann 1998). However, we did not obtain enrichment of GACA₄ or GATA₄ microsatellites in geoduck or eulachon (data not shown) at RT. All wash steps were performed without removing the sample from the 48°C environment. Using the MPC between washes, the three wash steps were performed as follows: 2×SSC+SNXf, 1×SSC+SNXf, 0.5×SSC+SNXf, using 200 µg

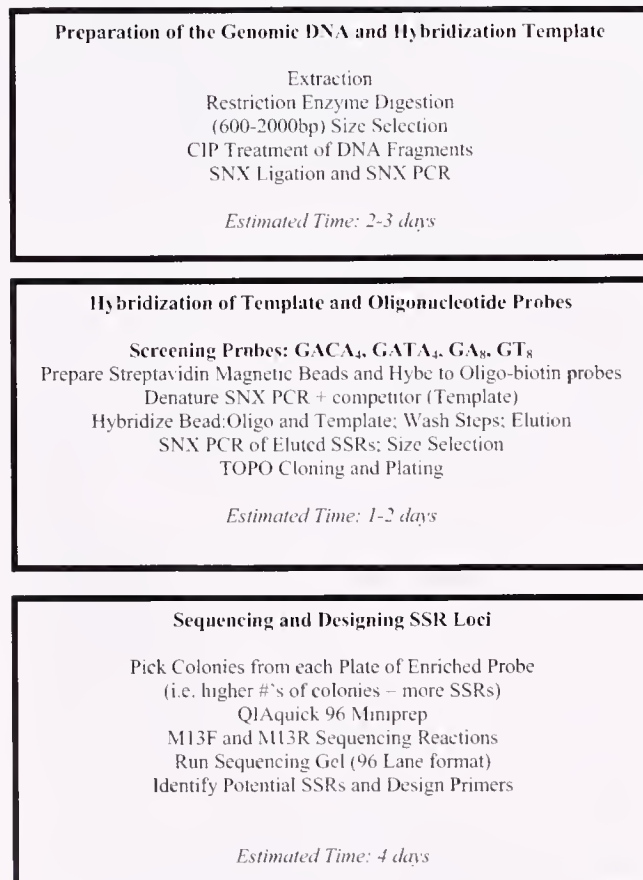


Figure 1. Flow chart of the magnetic bead enrichment protocol. Each major step is noted within the shaded boxes. The total elapsed time for the library experiment is approximately 7–9 days. The 96 well format greatly reduces the time and labour, thus offsetting the potential increase in consumable costs. In practice, one could produce successful library enrichment from a new species within two weeks.

SNXf per 1 ml of SSC. The presence of the SNX competitor in the hybridization and wash steps ensured a high level of stringency, thus reducing non-specific probe binding. The microsatellite-enriched fraction was eluted by adding 50 μ l of TE at 48°C to the bead:oligo:DNA incubated at 95°C for 15'. Using the MPC, the enriched fraction was removed without disturbing the streptavidin beads. The enriched fraction was used as the template in the SNX PCR, as noted above. By resolving the SNX PCR and oligo-PCR products on agarose gels, using each fraction (ligations, hybridisation, washes, elutions) as a template, the technical success was assessed (Table 1). Ideally, the smear of products amplified by the SNX primer declined with each wash step.

Following the SNX PCR with the enriched fraction as the template, the fragments were size selected (600–2000 bp), cloned and transformed using a TOPO Cloning 5' PCR kit (Invitrogen, Carlsbad, CA). The transformations were plated, grown overnight at 37°C and single colonies miniprep using a QIAprep 96 Turbo Miniprep Kits (Qiagen, Valencia, CA). Clones were sequenced using Big Dye Primer M13F and M13R Sequencing Kits (ABI, Foster City, CA). In general, the plates with the highest number of colonies correlated to the most abundant repeat motif. For the identification of unique or rare microsatellites, extensive probing from plates with fewer colonies was required.

Sequencing gels were electrophoresed on an ABI 377 automated sequencer using ABI Prism 377-96 Collection software (ABI). Sequences were viewed and base-called using Sequencing Analysis 3.4.1 (ABI). Sequencher software (Gene Codes Corporation, Ann Arbor, MI) was used to remove the vector, align and group sequences and highlight microsatellite repeats. PCR primers were designed for each potential microsatellite locus.

Over the past decade, we have isolated and applied microsatellite loci to population genetics studies of more than 20 marine fish and shellfish species. The substantial empirical data gained from these studies guides our ranking of microsatellite sequences for primer design in new species. Our criteria follows seven general rules (in order of sequence preference): Primers are designed to (1) All perfect tetranucleotide repeats of 10 to 35, (2) All perfect dinucleotide repeats of 15–45 (noting that those with over 25 repeats can contain substantial stutter), (3) Slightly imperfect tetranucleotide repeats of 13 or more, and (4) Compound di-di, tetra-tetra, and di-tetra repeats with little or no imperfections. Primers are generally not designed to (5) di/tri, tri/tetra or sequences containing stretches of more than five single nt, as these often result in 1-bp alleles, (6) Highly imperfect loci, as these often contain single base insertions or deletions, also resulting in 1-bp alleles, and (7) Tetranucleotide repeats containing three mononucleotides (e.g., AAAN). In general, if sufficient flanking sequence is available, three sense and three anti-sense primers are designed for each locus, which enables the detection of null alleles in the first round of screening and provides a variety of size ranges to choose from for the design of multiplexes. Because our population surveys generally contain sample sizes of 100 or more individuals, loci with high numbers of alleles are still considered potentially useful.

Polymerase chain reaction (PCR) amplifications were performed using 35 cycles of 94°C/30", 48–54°C/30", and 70°C/45". Each 8.0 μ L reaction contained 0.50 μ L of a 1:2 dilution of Chelex extracted DNA (approximately 0.01–0.03 μ g), 0.48 μ M of each primer, 0.80 μ M dNTP, 0.15 units of HotStarTagTM DNA polymerase (Qiagen, Valencia, CA) and 1 \times HotStarTagTM PCR Buffer containing Tris-HCl, KCl (NH₄)₂SO₄, 1.5 mM MgCl₂, pH 8.7. Primers were initially optimised by amplification of 8 individuals

size-fractionated on 10% non-denaturing polyacrylamide gels. Manual gels were stained with ethidium bromide, and sized against a 20-bp ladder using PhoretixTM 1D version 5.10 software (Non-linear Dynamics Ltd., Newcastle upon Tyne, UK). Multiple primers per microsatellite sequence were tested to aid in the identification of null alleles and to provide a range of product sizes for subsequent multiplexing. Null alleles and allele size ranges were assessed by amplifying 24 individuals for 2 to 3 primersets per polymorphic locus, size separated on manual gels. One sense primer for each polymorphic locus was then fluorescently labelled, and loci were incorporated into a multiplex for automated electrophoresis on an ABI 377 automated sequencer using 4.5% denaturing polyacrylamide gels. Allele sizes were determined with Genescan 3.1 and Genotyper 2.5 software (PE Biosystems, Foster City, CA), and the Genetic Data Analysis (GDA) program of Lewis and Zaykin (2001) was used to analyse allelic and genotypic frequency data.

RESULTS & DISCUSSION

The experimental time-table is outlined in Figure 1. The experimental design can be modified for high throughput, by employing a 96-well plate format MPC. This approach replaces the physically demanding, lengthy and potentially hazardous radioisotope method. The magnetic bead approach produced a high level of enrichment for tetranucleotide repeats in all three invertebrate species tested (Table 1). The microsatellite enrichment averaged 75% over all species/microsatellites surveyed. The level of enrichment of each microsatellite motif varied among species. In Dungeness crab, both GACA₄ and GATA₄ were highly enriched, at 85% and 82%, respectively. In geoduck clam, GACA enrichment was more successful (94% versus 45%) whereas in scallop, GATA was more highly enriched (92% versus 67%). Scallop was also highly enriched for CT₈, CCAT₄ and GACT₄ repeats.

Toth et al. (2000) demonstrated that di- and tetranucleotide motifs are the most common repeat in vertebrates. Epplen et al. (1998) compiled a table of representative microsatellite DNA sequences deposited in the EMBL/GENBANK data bank. They indicated that GAAA, AAAT, GATA and GGAA were the most common tetranucleotide repeats while GACA and GACT were rarer. Likewise, GT was the most common dinucleotide repeat followed by AT and GA. However, GATA and GACA were the most frequent tetranucleotide repeat in the bird, reptile and fish sequences surveyed (Toth et al. 2000). In human chromosomes, Katti et al. (2001) found tetranucleotide repeats were very frequent, with the most common type being (AAAN)_n. Indeed, Schable et al. (2002) obtained success in enriching for microsatellites from the dollar sunfish using (AAAG)₆ and (ACAG)₆. However, because AAAG repeats often contain imperfections such as AAG or AAAAG which result in 1-bp alleles, we did not enrich for (AAAN)_n microsatellites.

Herin, PCR protocols were optimised for tetranucleotide repeat containing microsatellites, which are generally found on the longer fragments. We note three steps in the enrichment process that were crucial to the obtainment of long fragments and sufficient flanking sequence. First, decreasing the SNX-ligation cycling steps from 18 to 5 cycles enhanced the length of flanking sequence. Second, increasing the number of cycles in the SNX-PCR from 30 to 40 enhanced the population of high molecular weight PCR fragments. Third, size selection of the 600–2000 bp fragments immediately following the restriction digest reduced self ligation of small frag-

TABLE 1.

Species used to test the enrichment protocol include geoduck (*Pab*), Dungeness crab (*Cma*), and Japanese scallop (*Pye*). All species were screened with GACA₄ and GATA₄ probes, with scallop probed for a variety of additional core repeats. The total number of clones sequenced, contained microsatellite repeats, and had primers designed are presented for each species. The % enrichment was determined by dividing the total number of microsatellite-containing clones by the total number of clones sequenced. Likewise, the % success was calculated by dividing the total number of useful loci by the total number of designed loci (primers for *Pye* have not yet been tested). Average insert length and average 3' and 5' flanking were calculated for each species.

Species	Probe Repeat	Number Sequenced	μsat Containing	Designed Loci	% Enrichment	% Success	Avg Insert Size (bp)	Avg 5' & 3' Flanking (bp)
<i>Pab</i>	GACA	33	31	10	93.94			
	GATA	22	10	3	45.45			
Total		55	41	13	74.55	30.77	401.2	103.3
<i>Cma</i>	GACA	87	74	18	85.06			
	GATA	67	55	0	82.09			
Total		154	129	18	83.77	50.00	448.9	129.6
<i>Pye</i>	GACA	48	32	4	66.67			
	GATA	48	44	10	91.67			
	CT	16	13	1	81.25			
	CA	16	9	2	56.25			
	CCAT	16	13	3	81.25			
	GACT	16	13	0	81.25			
	AAC	16	11	0	68.75			
	GGT	16	12	1	75.00			
Total		192	147	21	76.56	N/A	347	88.75

ments and bias towards smaller products in the PCR. Fourth, a second round of size selection of the 600–2000 bp fragments after the SNX-PCR was imperative because of the small insert bias of TA cloning. Using these protocols, we obtained an average fragment length of 347 bp (scallop) to 450 bp (crab) and average 5' and 3' flanking sequences ranging in length from 89 bp (scallop) to 130 bp (crab) (Table 1).

To directly assess the effectiveness of the enrichment protocol described herein, we compared the resolution of tetranucleotide repeat loci in geoduck clams obtained under the enrichment protocol to traditional library screening. Using traditional techniques, we screened over 3×10^4 clones using ³²P-end labelled (GACA₉) and (GATA₉) oligonucleotides, as in Miller et al. (2001). Sixty-six putative microsatellite-containing colonies were sequenced, 30 of which were found to contain microsatellite repeats. Only a single clone contained a simple tetranucleotide repeat. The remainder of the clones contained dinucleotide repeats, although four clones contained compound di-/trinucleotide repeats. Hence, although libraries were probed exclusively with tetranucleotide repeats, most microsatellites obtained were dinucleotide repeat sequences. Alternately, using the enrichment protocol, we sequenced 55 clones, 41 of which contained microsatellite repeats. Of these, 22 clones contained simple tetranucleotide repeats, 8 clones contained simple dinucleotide repeats, and the remaining 11 clones contained compound di-/tetranucleotide repeats. Hence, 80% of the microsatellite sequences isolated under the enrichment protocol contained tetranucleotide repeats versus 16% using the traditional method.

Primers to thirteen microsatellite sequences isolated through magnetic bead enrichment of geoduck clam were designed and tested. In addition, primers to ten microsatellite sequences isolated through conventional radioactive library screening (described

above) were designed. In all, one tetranucleotide (*Pab* 117) and three di-nucleotide loci (*Pab* 101f, *Pab* 132 and *Pab* 156) isolated through conventional screening were chosen for use in population studies, and four tetranucleotide repeat loci (*Pab* 101e, *Pab* 105e, *Pab* 106e, and *Pab* 112e) from the enriched method were chosen (Table 2). Loci were chosen by of degree of polymorphism, clarity of alleles, and absence of (demonstrated) null alleles defined in the original small survey of 24 individuals.

Polymorphism of the eight geoduck loci was evaluated over approximately 200 individuals collected from two sites within British Columbia, Canada, one located in the Strait of Georgia and the other off the Queen Charlotte Islands. The geoduck microsatellite loci were highly polymorphic, with numbers of alleles ranging from 21 (*Pab* 156) to 60 (*Pab* 132) and expected heterozygosities ranging from 0.91 (*Pab* 112e) to 0.97 (*Pab* 156). However, significant deviations from Hardy-Weinberg (HWE) equilibrium caused by heterozygote deficits were found at all but three of the loci (*Pab* 101e, *Pab* 106e and *Pab* 112e). Heterozygote deficiencies were also observed at most loci isolated from geoduck clams in a previous study (Vadopalas and Bentzen 2001), and have been commonly observed in a variety of marine invertebrate species (e.g. Brown 1991; Miller et al. 2001; Pérez-Losado et al. 2002; Addison and Hart 2004). Possible explanations for heterozygote deficits in marine invertebrate species include the presence of null alleles, a temporal Wahlund effect gained through Sweepstakes style recruitment (Hedgcock 1994), inbreeding, non-random mating, and selection.

Primers to eighteen microsatellite sequences isolated from Dungeness crab were designed and tested. Nine of these loci were chosen for use in population studies. Two loci contained dinucleotide repeat units (*Cma* 107 and *Cma* 118), six loci contained

TABLE 2.

Nine polymorphic microsatellite loci were developed from Dungeness crab and eight for geoduck clam. Repeats denoted with (i) contain imperfect repeated motifs. Locus-specific annealing temperatures are shown under T_a (°C). Levels of polymorphism measured by number of alleles and expected (H_e) and observed (H_o) heterozygosity, and inbreeding coefficients (F_{is}) were calculated from a survey of approximately 200 individuals collected at two sites for each species. The asterisk (*) under H_o denotes loci that contain a deficit of heterozygotes and are significantly out of Hardy-Weinberg Equilibrium. Accession numbers for each locus are shown in the end column. The first four geoduck clam loci were obtained through traditional library screening methods in which ^{32}P -labelled GACA and GATA probes were utilised.

Locus	Repeat	Primer Sequence (5'-3')	T_a (°C)	Size Range	No. of Alleles	H_e	H_o	F_{is}	Accession no.
<i>Dungeness Crab</i>									
<i>Cma</i> 102	(GACA) ₁₂	F: TTCAGCTGCACTTCAGTGAT R: CTGTAGTGAATAAATTACTGTT	50	136–175	10	0.75	0.77	−0.03	AY521552
<i>Cma</i> 103	(GACA) ₁₃	F: GTTCCAAATACAGTTGACC R: GTCTTCCTATGTCCTCCTT	48	205–226	9	0.71	0.70	0.01	AY521553
<i>Cma</i> 107	(GT) ₄₀	F: GCGTTCAAGGATATTACTGAGT R: GTTTCCTGACTCATCCCCCTC	50	145–220	39	0.92	0.69*	0.25	AY521554
<i>Cma</i> 108a	(GACA) ₁₃	F: GCAGTAGGAACAGCAGCTGAT R: GTTTATTTCGTCACCAGAGAGA	54	152–206	16	0.70	0.70	−0.00	AY521555
<i>Cma</i> 108b	(GACA) ₁₃	F: CAGGTGTGGTTGTGTCCTTTA R: GTTCAGTTGAACCCAGAGTGACA	54	116–137	8	0.71	0.72	−0.02	AY521556
<i>Cma</i> 114	(GACA) ₁₂	F: CAAGTAAGAGAATGGAATCGTATT R: GTTTGCCAAAGAGCATCAGTGACAA	52	233–257	7	0.61	0.55	0.09	AY521557
<i>Cma</i> 117	(GACA) ₉	F: GTCTGAGACGACCAACATC R: GTTTCACAGGAACATGAAATAGGAT	54	286–314	6	0.57	0.59	−0.05	AY521558
<i>Cma</i> 118	(GT) ₂₈	F: GGAGAGGGAGCGACTGTC R: GTTTGGTGTATTACAAAACAACAGTAA	52	167–203	19	0.85	0.88	−0.03	AY521559
<i>Geoduck Clam</i>									
<i>Pab</i> 101	(GA) ₁₆	F: TGTGTGAGATATAACCACTT R: GTTTGTCTATGGTTTGCATTGTA	56	80–215	27	0.94	0.52*	0.45	AY520562
<i>Pab</i> 117	(GACA) ₂₉	F: TGTGTGAGATATAACCACTT R: GTTTCGACCCAACAATAGTTGA	50	220–440	44	0.97	0.69*	0.28	AY520563
<i>Pab</i> 132	(AG) ₇₂	F: TCGCTACTAATCTCACTT R: GTTTGCTATTGATAATTCTGAGA	48	155–305	60	0.96	0.77*	0.19	AY520564
<i>Pab</i> 156	(GT) ₂₁ (CA) ₁₉	F: GAGTGACATAATGAGATACT R: TTTCATCTCGTTACATATCAATATT	50	100–250	21	0.91	0.62*	0.32	AY520565
<i>Pab</i> 101e	(GACA) ₂₀	F: GTACCTGATGGTGTAAATAGTA R: TTGATCATTATATTTTGTCTAGAC	52	119–282	23	0.93	0.86	0.08	AY520566
<i>Pab</i> 105e	(GACA) ₁₈ (GATA) ₅	F: CAACCATGGTGTCTCAAGA R: GGCAGATGGGTCTATAGTTT	50	128–214	21	0.93	0.72*	0.23	AY520567
<i>Pab</i> 106e	(GACA) ₂₄	F: GGCAGTCAGACAGACCAG R: ATGATCTCTCTATATCTGCTTCAAC	52	104–301	45	0.96	0.93	0.03	AY520568
<i>Pab</i> 112e	(GCAC) ₂₃	F: GCGCTTAGAATACTGCGGAAT R: GTTTACCATTACCATTTGTACCGTA	50	109–380	59	0.97	0.87	0.10	AY520569

tetranucleotide repeats (*Cma* 102, *Cma* 103, *Cma* 108a, *Cma* 108b, *Cma* 114 and *Cma* 117), and one locus contained a compound di-tetranucleotide repeat (*Cma* 1). Polymorphism of the eight loci was evaluated over approximately 200 individuals collected from two sites within British Columbia, Canada: Hecate Strait and Port McNeil. The microsatellite loci isolated from Dungeness crab contained a moderate level of polymorphism. The number of alleles identified at each of the loci ranged from 6 (*Cma* 117) to 39 (*Cma* 107) with an average of 14.4 alleles per locus. Expected heterozygosity ranged from 0.57 (*Cma* 117) to 0.92 (*Cma* 107). Deviations from HWE equilibrium was observed for only one of the loci (*Cma* 107).

In summary, the magnetic bead enrichment protocol described herein was successfully employed to isolate tetranucleotide repeat loci from marine invertebrate species that have been notoriously difficult to work with using traditional isolation methods. The

enrichment protocol can be performed in a few as 7 days, where as, standard methods can take months. The high throughput format enabled us to screen, clone and sequence hundreds of microsatellites, thus facilitating rapid isolation of new loci for multiplex population analysis. To date, we have successfully utilised this protocol to isolate microsatellite loci from six marine invertebrate (herein) and fish species (Kaukinen et al., in press and unpublished data).

ACKNOWLEDGMENTS

The authors acknowledge Tobi Ming, Karen Laberee, Shaocong Li and Brent Vadopalas for technical assistance. We thank Island Scallops for the Japanese Sea Scallops, and Johnstone Strait Dungeness Crab Fishery. Funding was provided by Fisheries & Oceans Canada and the Government of Canada, Canadian Biotechnology Strategy.

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ABSTRACTS OF TECHNICAL PAPERS

Presented at The 24th Annual

MILFORD AQUACULTURE SEMINAR

Milford, Connecticut

February 23–25, 2004

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OVERVIEW, 24th MILFORD AQUACULTURE SEMINAR.¹ Walter J. Blogoslawski, US Department of Commerce, National Oceanic & Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Ave., Milford, CT 06460

There were 164 registrants for the 24th Milford Aquaculture Seminar, a gathering of industry, research, and academic interests. By blending both the theoretical and practical aspects of aquaculture, the meeting permitted attendees an exchange of information concerning aquaculture methods outside their own expertise and provided a forum where the latest innovations were introduced and discussed.

Thirty-six formal papers and posters were presented by attendees from 11 US states, the District of Columbia, Brussels, Belgium, and Baja, Mexico. Meeting attendees represented 2 vocational aquaculture high schools, 10 universities, 4 marine laboratories, and several state and federal institutions involved in shellfish aquaculture. A highlight of the meeting was a set of papers reviewing the aquaculture research activities at the NMFS Milford Laboratory including immune response to toxic algal blooms, problems with growing large batches of microalgae, scallop culture, diseases that affect planted clams and oysters, and genetically enhanced farmed aquaculture products. Other papers covered state and federal impediments to siting aquaculture farms (NIMBY or Not In My Back Yard) and described how NOAA Sea Grant extension agents assist the aquaculture industry. Linda Chaves, NMFS Aquaculture Coordinator, detailed NOAA's position on aquaculture during a luncheon address.

A panel of scientists described the pitfalls and possibilities of introducing the Asian oyster, *Crassostrea ariakensis*, into Chesapeake Bay waters as a replacement for the native oyster, *Crassostrea virginica*, that has been devastated by disease. *C. ariakensis* has shown some potential as an alternative product for the ailing shellfish industry. Moreover, its introduction might help to restore ecologic function to diminished native populations. A careful methodical approach including the use of sterile triploid oysters is warranted.

At this year's seminar, representatives of 51 aquaculture companies gathered at an evening session for the annual meeting of the East Coast Shellfish Growers Association. The association's goals are to promote and protect shellfish members' needs in state and regional contexts and to involve all stakeholders in the task of enhancing the shellfish aquaculture industry.

The meeting was sponsored by the National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT. Abstract printing was courtesy of the US Department of Agriculture, Northeast Regional Aquaculture Center, N. Dartmouth, MA.

A NON-ELUCIDATED MORTALITY EVENT OF JUVENILE HARD CLAMS IN A HATCHERY SYSTEM. John Aldred,¹ Steven Pitchford,² and Bassem Allam³, ¹East Hampton Town Shellfish Hatchery, 159 Pantigo Road, East Hampton, NY 11937; ²USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; and ³Marine Sciences Research Center, Stony Brook University, Stony Brook, NY 11794

In the last few years, mortalities of juvenile bivalves sporadically occurred at the East Hampton Town Shellfish Hatchery (Fort Pond Bay, Montauk, NY), first in 1996 and again in 2001 and 2003. Histopathologic analysis performed on samples during the 2001 episode revealed heavy infestation by fungal mycelia in most tissues of dying animals. In the latest episode, batches of juvenile clams, generally about 1 mm in length, began to die off on May 19, 2003. An earlier batch of clams, which had been moved to a flowing water nursery system, seemed unaffected. Consistent with prior episodes, subsequent and apparently unaffected clam batches, if not moved to the nursery, would begin to die off in the hatchery juvenile systems usually as they approached 0.5 to 1 mm in length. Samples of juvenile clams, of different sizes and ages, some exhibiting symptoms and some not, were collected and submitted to standard histologic and staining techniques (hematoxylin eosin, Gram and GMS stains). Microbiologic analyses were performed on water samples taken from different parts of the system, but did not reveal any abnormal proliferation of heterotrophic bacteria. Histopathology analysis performed did not reveal any known pathogen in dying clams. The only consistent abnormal observation was the presence of chain-like microorganisms morphologically close to structures built by some colonial cyanobacteria, associated with some signs of starvation in affected juveniles. Whereas these structures were detected in four of five original samples (all 1 + mm in length), they were not detected in a fifth (0.3 mm) or subsequent sample (0.15 mm), both collected from conical tanks. The structures were found in larger clams after transfer to juvenile rearing tanks and seem to become more numerous with the progress of mortalities. Cyanobacteria of the genus *Planktothrix* and *Trichodesmium* have already been associated with bivalve mortalities, and microorganisms belonging to this group might be at the origin of juvenile mortalities. However, until the identity of the chain-like microorganisms is determined, the cause of the mortalities observed here remains unclear.

A FLOW-CYTOMETRIC METHOD FOR COUNTING MICROALGAL AND BACTERIAL CELLS IN THE SAME SAMPLE. Jennifer H. Alix and Gary H. Wikfors, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Numeric counts of microorganisms in aquatic samples often are essential to research and evaluation of aquaculture systems. Traditional methods of counting cells in the microscope or colonies on petri dishes are labor-intensive and subject to limitations in both

accuracy and precision. In water samples containing microalgae, bacteria, and other microorganisms, as well as non-living, suspended matter, proxy measurements, such as optical density, chlorophyll fluorescence, or packed-cell volume, are subject to error from interference of one particle type with another or inability to distinguish which types of particles are contributing to the measurement. Thus, particle enumeration often limits the temporal or spatial resolution of data. We have addressed this limitation with an admittedly sophisticated tool, the flow cytometer—not a tool expected to be available to most aquaculturists, but a versatile one if available!

The flow-cytometric (fcm) protocol we have been developing uses ratios of particles detected in the water sample with a known number of fluorescent, plastic microspheres added to the sample. Microalgal cells are differentiated from other particles by size and chlorophyll fluorescence measured in two detectors, and living particles have been differentiated from non-living by staining with several DNA-binding fluorochromes measured in a third detector. Identities of particles can be confirmed by physically sorting cells and observing them in the microscope.

Microalgal counts in both pure and open-tank (bacterized) cultures by our fcm method agree very well with microscope counts ($R^2 > 0.9999$) over three orders of magnitude (10^4 – 10^7), and bacterial counts from pure cultures agree reasonably well with plate counts. In open-tank microalgal cultures, clumping of bacteria in aggregates has made interpretation of counts difficult, but we are addressing this limitation with various anti-aggregating compounds with some success. Compared with plate counts, fcm counts of bacteria in microalgal tank cultures at least are in the same order of magnitude.

Processing time for each sample, including addition of reagents, fcm data acquisition, and data reduction, is approximately 3 minutes, which we estimate to be 1/3 the time for an algal microscope count alone. The labor saved is considerably greater if samples need to be settled and concentrated or diluted for valid hemocytometer microscope counts. The fcm method provides, in addition, some quantification of bacteria (subject to interpretation) and non-living particles with no further effort. We are applying this fcm, particle-counting method to studies of microbial and nutrient dynamics in open-tank, mass algal cultures.

THE STATUS OF QPX DISEASE IN NEW YORK. Bassem Allam,¹ Alistair Dove,² and Joshua Thiel³, ¹Marine Sciences Research Center, Stony Brook University, Stony Brook, NY 11794; ²Cornell University, Marine Sciences Research Center, Stony Brook University, Stony Brook, NY 11794; and ³New York State Department Of Environmental Conservation, Bureau Of Marine Resources, 205 N Belle Mead Road, East Setauket, NY 11733

The presence of the protistan parasite of hard clams quahog parasite unknown (QPX), was confirmed in different locations of Raritan Bay off the south shore of Staten Island during 2002.

Because of the importance of this clam population as a source for the hard clam relay fishery in New York State, we initiated a survey program to monitor the Raritan Bay population specifically as well as to assess the general distribution of QPX disease in hard clam populations from different locations of the marine district. Our results show that QPX is present among hard clams in Raritan Bay at relatively low prevalence in most surveyed sites (below 7%), but reaching higher levels in some cases (up to 30%). Seasonal changes in QPX incidence were noticed and a cyclical pattern of prevalence is suspected. There is some evidence that the prevalence of the infection is related to the density of wild clams in the field, with high QPX occurrence in areas with high clam densities. QPX was also detected at very low (background, <4%) levels in Oyster Bay and in the Peconic Estuary. Wild clams collected near transplant plots in the Peconic Estuary were negative with regard to QPX. To date, the only site displaying a high incidence of QPX (20%) in this estuary was found in Flanders Bay, a remote location from any transplant plot, suggesting that the presence of QPX in that site is unrelated to the transplant fishery. More data, however, are needed before drawing final conclusions. The QPX organism has been isolated from infected clams collected from Raritan Bay, and the molecular, physiologic, and ecological characterization of cultured parasites is in progress.

JUVENILE OYSTER DISEASE IN THE NORTHEAST: 2003 EPIZOOTICS RESULTED FROM INDEPENDENT ACQUISITIONS OF THE BACTERIAL AGENT. Katherine J. Boettcher and Aaron P. Maloy, Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, ME 04469

We have established the primary involvement of a *Roseobacter* species in juvenile oyster disease (JOD) in Maine, but prior to last year its potential role in other regions had not been verified. Further description of the bacterium is ongoing, but it has tentatively been named *Roseimarina crassostreae*. In 2003, samples of JOD-affected animals were obtained from locations in Maine, New York, and Massachusetts. The manifestation of the disease was similar at all locations (i.e. uneven valve margins and deposition of conchiolin), and the presence of these signs was correlated with extensive colonization by *R. crassostreae*. Previous characterization of isolates indicated that outbreaks of JOD result from a single infectious strain, and that once established the same strain can persist in JOD-enzootic systems year after year. These conclusions were supported by evidence obtained in 2003, which revealed that the Maine isolates were the same genotype as those isolated from the same location in the previous 3 years. Oysters from each epizootic in Massachusetts and New York were colonized by one of three strains of *R. crassostreae*. These could be differentiated from the Maine strains by differences in colony morphology and/or genetic markers. On the basis of these findings, we conclude that these outbreaks resulted from independent acquisitions of the bac-

terial agent at each of the affected locations. Continued development of management strategies should take into consideration that such potentially infectious environmental strains (rather than seed transfer) may be the most common source of new JOD epizootics.

COMMERCIAL CULTURE OF SOFTSHELL CLAMS HAS ARRIVED AND IS GROWING ON MASSACHUSETTS' NORTH SHORE. Joseph K. Buttner,¹ Mark Fregeau,¹ Scott Weston,¹ Bonnie McAneney,¹ Jack Grundstrom,² Anthony Murawski,³ and Evan Parker⁴. ¹Northeastern Massachusetts Aquaculture Center and Department of Biology, Salem State College, Salem, MA 01970; ²Ipswich Bay Cultured Clam Cooperative, 58 Railroad Ave. Rowley MA 01969; ³5 Upland Lane, Ipswich, MA 01938; and ⁴27 Turkey Rd., Ipswich, MA 01938

After a decade of restoration and enhancement efforts, two towns on Massachusetts' North Shore have initiated commercial culture of softshell clams, *Mya arenaria*. The Northeastern Massachusetts Aquaculture Center has supported these public and private initiatives with hatchery-reared clams.

Shellfishers in Ipswich and Rowley use similar techniques to capture wild spawn, seed sites and exclude predators. However, routes to commercialization differ. The Town of Ipswich supports a pilot study by two local, recent college graduates. In 2002, a 1.4 acre lease was approved for the bottom culture of softshell clams, the first such approval in nearly 40 years. Initially, wild caught seed were grown on prepared sites. Clams were captured and covered with 1/6" plastic webbing to exclude predators, predominantly the green crab (*Carcinus maenas*) and shorebirds. By summer 2003, many of the seeded clams had attained a legal size (>2 inches SL) and the first commercial harvest occurred. In excess of 40 bushels were harvested and marketed. In summer 2003, hatchery-reared seed clams ($n = 200,000$) as well as wild collected clams were stocked to enhance productivity of the leased flat. Town officials and culturists are assessing efficacy and refining protocols of the on-going pilot project.

The Town of Rowley initiated enhancement and restoration efforts on unproductive flats in the early 1990s, peaking with 800,000 hatchery-reared spat (12–17 mm) seeded annually in 2002 and 2003. The success of these culture efforts has generated enthusiastic support from town selectmen and the community at large. By fall 2003, the focus on public restoration evolved into a commercial endeavor. Four local clambers each obtained a 1-acre lease from the town for commercial production of softshell clams on tidal lands and then formed a cooperative. The appropriate permits to purchase and sell clams have been obtained. Permits for the harvest of cultured clams are pending, per modification by the Massachusetts Division of Marine Fisheries to coincide with established guidelines for wild clams from conditionally approved waters. The first harvest of marketable-size clams from leased flats is anticipated in summer 2005.

Over five acres of tidal flats are currently leased by North

Shore towns to community members for private aquaculture. Expansion to increase acreage, number of growers and to secure processing capabilities is being pursued. Success of the approach has not gone unnoticed as culturists from Kingston, MA; Oaks Bluff, MA; and Prince Edward Island, Canada have sought assistance to nurture public and/or private culture of softshell clams in their tidal waters.

GONAD MATURATION OF DELAWARE OYSTERS USING ARTIFICIAL FEEDS: AN EXPERIENCE WITHOUT CONTROLS. Walter J. Canzonier and Lisa M. Ragone Calvo, Maurice River Oyster Culture Foundation, Port Norris, NJ 08349

Controlled maturation of the gonad of Delaware Bay oysters outside the natural seasonal cycle has been problematic, and when this process has been pursued in the hatchery setting it has required the dosing of relatively small cohorts of potential spawners with massive quantities of cultured microalgae. In an effort to effectuate maturation out of season, a closed system was developed at the Haskin Shellfish Research Laboratory (NJ Agr. Expt. Sta., Rutgers University) in the winter of 1988, as part of a program to produce disease tolerant oyster seed on a commercial scale. A gonad maturation system (gonamaturator) was designed as a temperature controlled (22°C–24°C) side-loop of the main laboratory recirculated seawater supply. A shallow trough (4.8 × 0.9 m, operational pool depth 4 cm) was plumbed to permit continuous recirculation seawater at a rate of 34 L/min uniformly across its short axis via inlet and drain manifolds situated on the long axis. This configuration provided agitation to maintain the particles in suspension and afforded near uniform exposure of all the oysters in the trough. The water passed through an aeration/constant head column on each cycle through the loop and was continuously dosed (1 L/h) with a concentrated suspension of a specially formulated composite particulate feed via a diaphragm dosing pump and in-line mixing chamber prior to entering the inlet distribution manifold. The feed suspension consisted of a mixture of commercial corn starch, kaolin (extra fine grade) and a commercial microcapsule larval shrimp diet (Fripak Booster) at a daily rate of 19g, 11g, and 45g, respectively, per 50 to 65+ oysters. The suspension was prepared daily in cold freshwater, and the continuously stirred reservoir was maintained in a refrigerator at 4°C to minimize bacterial degradation. The side loop operation was continuously controlled by a programmer constructed of inexpensive and readily available electronic components. Hourly, the feed dosing pump was stopped for a 15 minute period to allow the oysters to exhaust the suspended feed; the trough and associated piping were then drained back to the main laboratory seawater system. The gonamaturator was then refilled to operating capacity, after which the circulation/feeding cycle was restarted. Activity of the oysters was monitored daily, and was judged to be optimal on the basis of pumping activity and fecal string production. Two lots of disease tolerant parent stock were conditioned to maturation in 38 and 18 days, respectively,

with minimal mortality during the maturation phase, and good spawning in a contracted commercial hatchery. Competence of the eyed larvae was judged satisfactory, as judged by eventual setting efficiencies in the range typically obtained in our remote setting operation. Previously, algal paste from two sources had been tried for conditioning, but with mixed results. Acceptance of this type of feed was poor to fair at best, as indicated by activity levels and feces production. Indeed, one batch of slurry from a commercial source completely inhibited opening and pumping by the majority of the oysters. Algal paste was eventually abandoned as a feasible alternative.

This project was partially funded by USEPA, Region III, Delaware Estuary Program Grant 66.456, a Delaware River & Bay Authority Industry Development Grant, and the New Jersey Sea Grant/Marine Science Consortium.

TRACKING LAB-BRED BAY SCALLOP GROWTH AND SURVIVAL IN THE NANTIC RIVER. Joseph Choromanski, Sheila Stiles, Dorothy Jeffress, Ronald Goldberg, and David J. Veilleux, USDOC, NOAA National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Selection efforts on the bay scallop, *Argopecten irradians irradians*, have provided laboratory-reared lines that allowed us to develop strategies for studying certain characteristics, such as growth and survival, in restoration projects. These strategies include increasing the frequency of phenotypic markers of striped shells for stock identification in enhancement and restoration efforts while maintaining or even increasing survival and growth rates. Over the course of two seasons, trials have been underway in the Niantic River in Connecticut with these stocks of scallops.

Scallops with and without striped shells were deployed in three tiered cages at two sites in the river. Cages were made of plastic-coated wire with a 7.5 cm mesh. Each cage measured 56 × 56 × 94 cm and was divided horizontally into three sections or tiers. Cage inserts of smaller mesh (10 and 17.5 mm) measuring 41 × 10 × 81 cm were used to hold the scallops. The initial year's experiment was deployed for growth and survival at densities of 100 scallops per tier with an equal number of striped and non-striped scallops from the same genetic lines. The cages were checked monthly to the end of the normal growing season to determine performance, with the added attention to shell deformities that might indicate density problems. Fouling organisms, which may prevent adequate water flow through the cages, were checked for and removed. Overwintering trials were then carried out to compare survival into the next growing season with the aim of utilizing the striped scallops as brood stock for restoration trials the following summer. Growth trials of a second line of striped scallops repeated these efforts during the second season. Preliminary data have shown favorable indications and will be compared with past studies at different sites and in lines maintained at the laboratory.

OBSERVATIONS ON PHENOTYPIC PLASTICITY AND BEHAVIOR IN A GENETIC LINE OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS IRRADIANS*. Allison Clark,¹ Sheila Stiles,² Dorothy Jeffress,² and Joseph Choromanski,² ¹University of Rhode Island, Kingston, RI 02881; ²USDOC, NOAA National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Organisms may change their physical characteristics or behavior to escape predators, a feature that can be known as phenotypic plasticity or inducible defense mechanism. A laboratory experiment was conducted using a genetic line of bay scallops, *Argopecten irradians irradians*, to test for phenotypic plasticity. Responses were measured because changes in morphometric traits and avoidance or attachment behavior caused by exposure to effluent from predators and damaged conspecifics. Six hundred juvenile scallops with and without striped shells from a mass-spawned genetic line were divided equally among 3 treated or exposed groups and 3 replicate controls in aquaria with flowing seawater. Six crabs, 3 each of green and mud crabs—predators of scallops in the wild—were placed in a cage inserted into a headjar and fed crushed scallops. These damaged conspecifics were from the same genetic line as the experimental scallops. A second headjar, similar to the treated experimental apparatus but without crabs, was used for the controls. The results of this experiment did not show significant difference between treated and control scallops for changes in total weight, shell weight, or shell length, following an effluent exposure period of 36 days. There also was no significant difference between scallops with and without striped shells in response to the effluent exposure or waterborne cues. It was observed, however, that the juvenile scallops were more likely to attach to the vertical sides of the tank when exposed to the effluent. This result highlights the importance of a vertical substrate, such as eelgrass, to the survival of young bay scallops.

A REVIEW OF LEGAL AND POLICY CONSTRAINTS TO AQUACULTURE IN THE US NORTHEAST. John A. Duff,¹ Tessa S. Getchis,² and Porter Hoagland³, ¹Environmental, Coastal and Ocean Sciences Department, University Of Massachusetts, Boston, MA 02125; ²Connecticut Sea Grant, 1080 Shennecossett Road, Groton, CT 06340; ³Marine Policy Center, Woods Hole Oceanographic Institution, Woods Hole, MA 02543.

Throughout the United States, aquaculture firms face a wide variety of laws and regulations that govern the manner in which they plan, site, and operate aquaculture facilities. It is obvious that aquaculture cannot be conducted in the absence of a legal system that clarifies or establishes property interests, provides a means for the protection of these interests, and ensures the safety of the product for consumers.

Although a legal framework is necessary for aquaculture to exist as an industry, there are many instances where uninformed, outdated, or inappropriate regulatory regimes impede aquaculture

development. Inconsistencies in the law can lead to an uncertain legal environment for aquaculturists. Regulators often are put in the conflicting position of promoting the development of the industry and regulating its effect on other uses of the land and sea. Firms are sometimes forced to undertake activities while lacking adequate information or a complete understanding of laws and regulations. Conflicts and concerns may be left unresolved until an issue is brought before an adjudicatory body. Legal constraints such as these detract from the stability and certainty that otherwise would facilitate sustainable aquaculture development, slowing or halting the growth of the industry, or perhaps even leading to its decline.

During the summer of 2003, we reviewed a range of aquaculture policies in an effort to identify those laws and regulations that may impede development unnecessarily within the northeastern United States. Through an informal survey of industry and government officials and a review of the literature, we find that specific laws and policies or the absence of laws and policies can be argued to impose constraints on growth in certain segments of the industry.

The northeastern "aquaculture industry" comprises a set of markets that may or may not be closely linked or even share technologic approaches. Typically, we conceptualize the structure of a market as a "vertical" flow of product from hatchery to grow-out to the downstream activities of processing, distribution, and final retail sale to the consumer. Firms may or may not be vertically integrated from production through retail. A number of separate markets exist for individual species of fish or shellfish. Some of these markets may be closely related; for example, blue mussels and hard clams are economic substitutes. Others may be very distinct, as baitfish are not close substitutes for farm-raised trout. Another cross-cutting issue concerns production technologies, which might be similar across markets but could differ within the same market.

Market and technologic factors could influence the extent to which a particular law or policy is perceived as a constraint. As a hypothetical example, a firm that processes cultured shellfish might like to see more farm production, which would reduce the price that it pays for raw product. Thus, this firm, situated downstream in the processing sector of the industry, might argue that riparian interests limiting the number of tideland leases are a clear constraint to industry development. On the other hand, firms with existing leaseholds might prefer that it be difficult for competitors to obtain access to additional areas because more production could reduce their own revenues. The extent to which these considerations are valid depends upon competition in the market (i.e., are producers price-takers who are selling their product at marginal cost?). These are the kinds of issues that should be kept in mind when thinking about the extent to which laws and regulations are truly constraints in a broadly defined US northeastern aquaculture industry.

We have identified 11 policy and legal issues that likely con-

strain the growth of the industry. In rough order of importance, these issues include: administrative and jurisdictional overlaps, lease and tenure processes, control of disease, interstate transport of product, competition with foreign imports and international trade barriers, policies governing interactions with protected species or impacts on habitats, rules concerning the culture of commercially harvested species, federal and state effluent regulations, culturing of genetically modified organisms, culturing non-indigenous species, and permitting in the US exclusive economic zone.

The most important potentially constraining issue concerns administrative complexity (often referred to as administrative "overlap"), where confusion about the relevant rules may lead to excessive financial and time costs. It is important to recognize that criticisms of administrative overlaps sometimes are inapt in that there may be no explicit "overlap" in the strict sense of two agencies regulating the same activity. In discussions with both industry participants and government officials, we have found that the term "overlap" often is used in a general sense to connote the complexity of regulation and the confusion that is the predictable consequence of that complexity.

A review of state statutes governing the designation of aquaculture "lead agencies" suggests that northeast states have made substantial efforts in recent years to respond to concerns about jurisdictional overlap. The recurring identification of administrative overlap as an industry impediment may be one of perception as much as effect. Nonetheless perceptions may have real impacts and, as such, states might consider efforts that would characterize aquaculture-related laws and regulations as residing in one "place."

Issues relating to interstate trade are perceived by many in the industry to be among the most constraining. In the future, research might usefully be directed at characterizing the laws and policies among the states of the northeast region that relate to animal health, including disease inspections and certifications. Also, state laws and regulations that restrain trade on the basis of conserving and managing wild harvest fisheries ought to be made clearer. Increasing the transparency of these rules and highlighting non-uniformities among disparate state approaches to the control of movements of diseases and invasive species, as well as to conservation of fishery resources, is likely to lead ultimately to a more homogeneous and less confusing regional legal regimen.

We also heard interest voiced by the industry in the development of so-called best management practices (BMPs). There are a number of precedents in other regions or internationally, such as the aquaculture industry in Europe, where BMPs have been adopted and implemented by participants in specific markets. A perceived advantage of the BMP approach is that it emerges as the product of consensus among industry participants, sometimes guided by government agencies. As such, BMPs are a form of soft law, a set of normative principles that does not involve explicit regulation. It would be productive to direct legal and policy re-

search toward understanding the implications of BMPs for market structure, their effectiveness in achieving stated objectives, their costs in comparison with government regulation, and their resiliency over time.

A REPORT ON THE 2003 CONNECTICUT SHELLFISH INDUSTRY SUMMIT. Tessa S. Getchis, Connecticut Sea Grant, 1080 Shennecossett Road, Groton, CT 06340

In November of 2003, individuals representing 30 commercial aquaculture businesses, several industry groups and associations, and members of Connecticut Sea Grant Extension, met to discuss the status of Connecticut's shellfish industry. The goal of the meeting was to identify the key challenges facing the State's industry, and to propose methods for addressing the challenges through collaborative research and cooperation with other stakeholders. The objectives of the meeting were: (1) to develop a list of challenges and needs (rationale for solving industry problems, grant development, etc.); (2) to prioritize challenges and needs (high, medium, or low priority); and (3) to draft a report of the discussion and conclusions. The report was drafted to solicit input and assistance from industry associations, councils, university researchers, educators, legislators, regulators, etc.

The participants of the summit ranked challenges to the shellfish industry in order of importance. Among the challenges ranked high were: (1) permitting restrictions on submerged and/or floating shellfish cultivation gear; (2) a confusing regulatory environment; (3) interaction with competing user groups in Long Island Sound; (3) increase in non-water based uses of the coastal zone resulting in lack of adequate workspace for industry; (4) state natural oyster beds have not been revitalized in several years; and (5) perceived negative impact on the industry resulting from the merger of the State Departments of Agriculture and Consumer Protection. Those challenges, which ranked medium to high, were: (1) interaction between eelgrass and shellfish cultivation gear; (2) oyster disease; (3) increased competition in shellfish markets, depressed prices for product; and (4) negative public perception of aquaculture in the state. Of medium importance were: (1) lack of industry leadership and (2) intrusion of energy line crossings in Long Island Sound. Of low to medium importance was limited amount of applied aquaculture research at Connecticut universities. Challenges ranked low by the participants included: (1) impact of invasive and native fouling organisms on gear, leading to increased maintenance costs; (2) inconsistent regulations for interstate transport of seed and product; and (3) increased fishing pressure on hard clams.

ENVIRONMENTAL CONDITIONS AND EXPERIMENTAL INFECTIONS WITH *P. MARINUS* MODULATE CELLULAR DEFENSE MECHANISMS IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. Michael Goedken and Sylvain De Guise, Department of Pathobiology and Veterinary Science, University of Connecticut, 61 North Eagleville Road, U-89, Storrs, CT 06269

The fast growing oyster aquaculture industry is greatly hindered by *Perkinsus marinus* and *Haplosporidium nelsoni*, which can kill up to 80% of production. The relationship between parasites, oyster defense mechanisms and their environment is unclear. Phagocytosis (using fluorescent beads) and apoptosis (using Annexin-V FITC) of eastern oyster (*Crassostrea virginica*) hemocyte subpopulations were quantified at the single cell level utilizing flow cytometry. The influence of salinity (428 mOsm or 1.006 vs. 1006 mOsm or 1.025) and temperature (11°C vs. 25°C) were evaluated experimentally. Forward and side scatter distinguished two populations of hemocytes (granulocytes and hyalinocytes) with previously demonstrated unique functional characteristics. Controlled environment and infection studies revealed significant immune function alterations. Phagocytosis in granulocytes, but not in hyalinocytes, was higher at low salinity (428 mOsm or salinity of approximately 1.009) than at high salinity (1006 mOsm or salinity of approximately 1.025) when the water temperature was 25°C. Phagocytosis was not affected by salinity when the water temperature was 11°C. Temperature had no effect on hemocyte phagocytosis at either salinity. Apoptosis in both granulocytes and hyalinocytes was higher at 11°C than at 25°C when the water salinity was low (428 mOsm). Temperature had no effect on hemocyte apoptosis when salinity was high (1006 mOsm). Salinity had no effect on hemocyte apoptosis at either water temperature. Apoptosis in both granulocytes and hyalinocytes was higher upon *in vitro* infection with *P. marinus* compared with uninfected control cells. We demonstrated experimentally that water salinity, water temperature, and experimental infection with *P. marinus* modulated oyster defense mechanisms. A better understanding of how these variables affect oyster defense mechanisms may lead to management strategies that will result in reduced disease morbidity and mortality for oyster producers.

MONITORING SUCCESS OF BAY SCALLOP RESEEDING AS PART OF THE NORTH CAPE OIL SPILL SHELLFISH RESTORATION PROGRAM. Heidi J. Green,¹ Karin A. Tammi,² and Michael A. Rice³, ¹Department of Marine Affairs, University of Rhode Island, Kingston, RI 02881; ²Ridem Division Of Fish and Wildlife Coastal Fisheries Laboratory, Wakefield, RI 02879; ³Dept. of Fisheries Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881

On January 19, 1996, during a storm, the *North Cape* barge and the tugboat *Scandia* grounded off the coast of Moonstone Beach in South Kingstown, Rhode Island. The barge spilled approximately

828,000 gallons of No. 2 home heating oil into Block Island Sound and surrounding tidal waters. Large numbers of crustaceans, mollusks, birds, amphipods, and fish were killed due to the oil spill. A legal settlement resulted, requiring the responsible parties to compensate for restoration, including \$1.5 million allocated to shellfish restoration using bay scallops, *Argopecten irradians irradians* (Draft Shellfish Restoration Plan and Supplemental Environmental Assessment for the North Cape Oil Spill [DSRP], 1999). In September 2002, the Trustees (Rhode Island Department of Environmental Management (RIDEM), National Oceanic and Atmospheric Administration [NOAA], and the US Department of the Interior [DOI]) initiated a bay scallop restoration program by re-seeding Point Judith Salt Pond. An estimated 679,640 of 25-mm multicolored bay scallops were seeded near major eelgrass beds at a density of 0.84 scallops/m². Seeding encompassed an estimated 805,000 m² and was restricted to scallop habitat in the midsection of the pond. In the summer 2003, a study was conducted to evaluate the re-seeding effort by using artificial spat collectors. A total of forty longlines and 800 spat collectors were deployed over four study sites in the pond. Scallop recruitment to the collectors was very poor ranging from only 0.01 to 0.158 scallops/collector; indicating that the Fall 2002 scallop seed did not survive to “throw a spawn” in the summer of 2003.

IMPROVING THE EASTERN OYSTER BY INTERSTRAIN HYBRIDIZATION. Ximing Guo,¹ Susan Ford,¹ Gregory DeBrosse,¹ and Roxanna M. Smolowitz,² ¹Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349; ²Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543.

The eastern oyster industry in the Northeastern region faces three major diseases: MSX (caused by the parasite *Haplosporidium nelsoni*), Dermo (caused by the parasite *Perkinsus marinus*), and the juvenile oyster disease (JOD, probably caused by a bacterium). Rutgers University has been breeding oysters for disease-resistance since the early 1960s and produced strains showing strong resistance to MSX and some resistance to Dermo. Breeding at the F.M. Flower Oyster Company has produced a strain (FMF) showing superior growth and JOD-resistance. As part of a project funded by NOAA's Oyster Disease Research Program, we produced and evaluated the Rutgers NEH strain, the FMF strain and their hybrids (HYB) along with a global susceptible control (ME) and local controls that were normally cultured at each of the four deployment sites in NJ (2), CT, and MA. Oysters were produced in June 2000, deployed in July 2000, and evaluated for 27 months. Dermo exposure was heavy at most sites, whereas MSX and JOD infections were low or absent. At Cape Shore (NJ) where infection was the heaviest, NEH and HYB had the lowest cumulative mortality, 43.5% and 43.6% respectively, compared with 82.3% for FMF, 99.4% for ME, and 81.1% for the local control (Delaware Bay wild). Mortality patterns were in general agreement with

Dermo infection levels observed. In growth, HYB was the same as FMF and faster than NEH, whereas ME and the local controls grew the slowest. The hybrid offered the highest yield by surviving as well as the NEH strain and growing as fast as the FMF strain. Despite some variations, data from other sites showed the same pattern in growth and survival.

IMMUNE RESPONSES OF TWO POPULATIONS OF THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, TO NATURAL AND SIMULATED BLOOMS OF THE DINOFLAGELLATE, *PROROCENTRUM MINIMUM*. Hélène Hégaret and Gary H. Wikfors, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Recurring summer blooms of the dinoflagellate, *Prorocentrum minimum*, have been observed for decades in Lagoon Pond, Martha's Vineyard, MA. These blooms seem to interfere with shellfish aquaculture production, both in the Martha's Vineyard Shellfish Group (MVSG) hatchery and in the pond. In cages suspended from the MVSG dock in Lagoon Pond, we placed juvenile oysters, *Crassostrea virginica*, from 2 sources: (1) progeny of broodstock maintained in Lagoon Pond for many generations (“native”) and (2) seed from a Maine hatchery where *Prorocentrum* blooms have not been observed (“non-native”). Oysters were analyzed for cellular immune-system profiles using flow-cytometric analyses for hematologic characteristics and several hemocyte functions. Hemocyte measurements were made after several weeks of acclimation and during two *P. minimum* “bloom events”—one simulated by the addition of cultured *P. minimum* to natural plankton in basins with flowing seawater, and the other naturally occurring.

For both populations of oysters, *P. minimum* exposure had a significant effect upon immune profile. Moreover, natural and artificial blooms of *P. minimum* triggered a similar immune response in the oysters, indicating that the artificial bloom was a good simulation of a natural bloom. “Native” and “non-native” oysters differed significantly in one key immune-function parameter—hemocyte oxidative burst. This difference between the two oyster populations suggests that the native oyster population may have been selected genetically for a more effective defense response to *P. minimum*, because invertebrates do not have an adaptive immune system by which an individual can improve its response following initial exposure to a pathogenic or toxic organism. These findings also offer clues to possibly explain differential survival of “wild” and cultured bivalves in waters experiencing harmful algal blooms.

SARGENT'S COVE, A CONNECTICUT SHELLFISH HATCHERY. Laura Ibars, Erin McManns and Javier Saenz De Pipaon, Sargent's Cove of Connecticut LLC, 132 Water Street, South Norwalk, CT 06854

Sargent's Cove of Connecticut is a new shellfish hatchery located in South Norwalk, Connecticut and a nursery in Darien,

Connecticut. The company is focused on the American oyster, *Crassostrea virginica*, and the hard clam, *Mercenaria mercenaria*, with a production goal for 2004 of 50–100 million oysters.

Five types of algae are grown at the hatchery for broodstock, larvae, and post set oysters: Brown algae (CCMP609 and T-Iso); green algae (PLY429); and diatoms (3H and Chaet).

The broodstock oysters are collected from different farmed oyster beds of Hillard Bloom Shellfish and are conditioned for approximately 6 weeks. The larvae are drained every 2 days until ready to set and then are moved to setting silos (using a downweller system) with clamshell cultch.

The land-based nursery has 12 tanks used as an upweller system where fresh water from Long Island Sound is constantly pumped, although no algae are added to the juvenile oysters then. The only work at this point is sorting and cleaning. Once the juveniles are larger than 1 mm they are moved to the FLUPSY, powered by electricity and tides. Here they will stay until they attain at least 25 mm in size. Once they are big enough they are planted on Hillard Bloom Shellfish farming grounds for 1 or 2 years after which commercial size is attained and the product is ready for market (at least 75 mm in size).

RE-CLAM-ATION OF UNDERWATER LANDS: THE NATURE CONSERVANCY'S EFFORTS TO RESTORE THE GREAT SOUTH BAY, NEW YORK. Carl Lobue, The Nature Conservancy Long Island Chapter, 250 Lawrence Hill Road, Cold Spring Harbor, NY 11724

In December 2002, in the largest transaction of its kind, the parent corporation of the Bluepoints Company transferred 11,500 acres of submerged land in New York's Great South Bay to The Nature Conservancy (TNC). This underwater land holding, which covers about 20% of the underwater lands in Great South Bay, provides an unprecedented opportunity to explore the effectiveness of a variety of alternative strategies for restoring key ecologic targets such as hard clam populations and seagrass communities. The Conservancy has formed a coalition of resource managers, scientists, and stakeholders called the Bluepoints Bottomlands Council to help formalize a long term management plan for these underwater lands and to assist with the development and implementation of system-wide restoration initiatives. Hard clams are the group's first ecologic target.

Hard clams are a keystone species in the bay. As nature's water filters, healthy stocks of suspension feeding bivalves, particularly long-lived species such as clams and oysters, are ecosystem engineers that are critical for maintaining a stable plankton community. After the loss of native oyster stocks in the last century, hard clams became the dominant suspension-feeding bivalve in the system, filtering 40% of the bay daily. Hard clams were also a principal component of the benthic community, creating structure and transporting pelagic production to the benthos.

Hard clam populations in Great South Bay have undergone an

extraordinary decline over the past 30 years reducing annual harvest rates by over 98% from their peak in the mid 1970s. It is widely acknowledged that the initial stock declines were a result of unsustainable harvest; however, in recent years the relative exploitation rates have declined whereas recruitment has continued to remain poor. This poor recruitment seems to be a result of low and sparsely dispersed spawning stock combined with poor overall food quality. Despite the aquaculture activities by the Bluepoints Company, the property it formerly owned had fallen to the same fate as the adjacent public lands. The hydraulic escalator dredges it used to harvest clams resulted in a clam population that is an order of magnitude lower than that on the depleted public lands (averaging 0.18 legal-sized clams per square meter in formerly productive areas). The current population of hard clams in the bay is estimated to filter about 1% of the bay daily and clams have not been replaced by other suspension feeders.

The Conservancy recently received support from the NOAA CRP to establish a network of spawner sanctuaries on TNC underwater land in Great South Bay. This effort will also monitor all the life stages in these areas from the survivability and spawning of stocked clams, into the planktonic stages through recruitment. Future plans include working with the towns, county, state, and federal agencies to consolidate existing information, coordinate bay-wide surveys, develop and implement a bay-wide spawner sanctuary plan, and protect new clam sets that occur on TNC property. In the long term the Conservancy hopes to foster system-wide shellfish management strategies that will promote sustainable harvest rates and support adequate shellfish abundance to maintain a healthy ecosystem function.

ASSESSING SHELLFISH AQUACULTURE ALONG THE EAST COAST. Sandra Macfarlane¹ and Gef Flimlin², ¹Coastal Resource Specialists, PO Box 1164, Orleans, MA; ²Rutgers Cooperative Extension, 1623 Whitesville Road, Toms River, NJ 08755.

The East Coast Shellfish Growers Association determined that developing best management practices for East Coast shellfish aquaculture was a high priority. As a prelude to that effort, an important step was to gain an understanding of the extent of the industry. The authors surveyed the Atlantic coastal states to investigate the number and size of leases, total acreage under lease agreements, species cultured, methods used, number of people unemployed, regulatory framework, user conflicts, and local issues. Results confirmed anecdotal evidence that the industry along the East Coast is comprised of many individuals farming relatively small leases (5–10 acres). Nearly 3,000 leases are currently in use for over 50,000 acres (excluding VA that has 100,000 acres leased but few acres currently under cultivation). Oysters and hard clams are the principal species, regulatory frameworks have many commonalities. The next challenge will be to find commonalities among this diverse industry to develop a meaningful code of prac-

tice and some sort of management system that will encourage the industry to thrive and grow but will also protect the public waters in which the leases are held in the public trust.

HOW INVERTEBRATE ABUNDANCES CORRELATE WITH QUAHOG ABUNDANCES IN FOUR BAYS IN THE NORTHEASTERN UNITED STATES. Clyde L. Mackenzie, Jr., USDOC NOAA Northeast Fisheries Science Center, James J. Howard Marine Sciences Laboratory, 74 Magruder Road, Highlands, NJ 07732

The abundance of quahogs, *Mercenaria mercenaria*, differs widely in four bays in the northeastern United States. Point Judith Pond in Rhode Island and Raritan Bay in New York–New Jersey have abundant quantities of quahogs, but Great South Bay in New York and Barnegat Bay in New Jersey now have relatively sparse quantities. Further, the meats of quahogs in Great South Bay and Barnegat Bay commonly are gray rather than the preferred creamy-white, and in Great South Bay quahogs grow slowly and their meats are thin. Great South Bay and Barnegat Bay once had large abundances of quahogs and were large quahog producers. Fishermen harvest quahogs in the four bays using mostly bull rakes, but also tongs. Harvesting pressure on the quahogs is relatively heavy in Point Judith Pond and Raritan Bay, but due to low abundances, harvesting is light in Great South Bay and Barnegat Bay.

This study was conducted to determine whether data on the abundances of the benthic invertebrates associated with the quahogs in the four bays would aid in determining the causes of the quahog declines in Great South Bay and Barnegat Bay. If the other invertebrates were also relatively scarce in the two bays, then it was presumed to be unlikely that overharvesting was the primary cause of the declines in quahog abundances. In 2003, the invertebrates in the four bays were sampled with a 6×6 cm hand grab. All four bays had a broad array of invertebrate groups: mollusks, crustaceans, polychaetes, and nemerteans. Nearly all were juveniles. Point Judith Pond and Raritan Bay had an average of about 1,200 invertebrates/m², whereas Great South Bay and Barnegat Bay had significantly fewer: 186 invertebrates/m² and 365 invertebrates/m², respectively. It is concluded that over-harvesting did not lead to the quahog declines in Great South Bay and Barnegat Bay, but rather they probably were mostly due to the effects of brown tides (caused primarily by *Aureococcus anophagefferens*) and perhaps other detrimental components in the water and to predation by blue crabs, *Callinectes sapidus*. Other factors besides brown tides may contribute to poor water quality in the bays, because resident times of water in Great South Bay and Barnegat Bay can be at least 2 months. Brown tides and blue crabs have become abundant in the two bays during the past 20 years, but neither are abundant in Point Judith Pond and Raritan Bay. In Great South Bay and Barnegat Bay, poor water quality, in part due to the brown tides, most likely prevents normal gamete production, larval development, and growth of the bays' invertebrates. Also,

after their larvae settle blue crabs eat most of the invertebrates, including quahog seed.

THE SLOW DEVELOPMENT OF CULTURE (AQUACULTURE) OF SHELLFISH AND FINFISH IN ESTUARIES OF EASTERN NORTH AMERICA AND ITS FUTURE. Clyde L. Mackenzie Jr., USDOC, NOAA Northeast Fisheries Science Center, James J. Howard Marine Sciences Laboratory, 74 Magruder Road, Highlands, NJ 07732

The commercial culture of bivalve shellfish and finfish has proceeded slowly and is not likely to expand much in the eastern estuaries of Canada, the United States, and Mexico. The principal reason for this is that estuarine shorelines and waters have been increasingly taken over by residential and industrial development, piers and boats. The culture described is the rearing of young stages in privately-owned hatcheries followed by growout of the young to market size in leased estuarine plots (aquaculture). Other than *Mercenaria mercenaria*, of which an estimated 20% of total United States production is from hatchery-growout culture (Milford hatchery-VIMS grow-out methods), this type of production of *Crassostrea virginica*, *Mya arenaria*, and *Argopecten irradians* is minute when compared with that of wild set. In Canada, shellfish aquaculture is practiced on a limited scale whereas salmon production has reached significant levels.

The reasons for the low production of such-cultured shellfish are (1) space to operate hatcheries has been limited by high costs of shorefront land; (2) growout on beds is also limited because non-hatchery shellfish are present in most otherwise suitable areas and thus they are unavailable for leasing; (3) environmental regulations are strict; (4) some otherwise available sites are polluted; (5) home, industrial and marina owners, and recreational boaters whose numbers are increasing rapidly, have found shellfishing operations (stakes and floats and men working in leased areas) objectionable; (6) large natural sets of shellfish on public and leased beds occur frequently and after they grow they are highly competitive in markets; and (7) consumer demand for shellfish is limited.

The culture of finfish, whose market demand is strong, has been nonexistent except for salmon in Canada and Maine. There are a number of reasons for this. Some are biologic/ecologic such as unfavorable extremes of temperature that limit the growth and survival of many potential aquaculture species. Others are logistical such as severe storms that threaten costly nets and cages. Still others impact the economics of enterprises such as the high cost of fish feed and other basic materials, to say nothing of high labor costs in these regions. Moreover, consumers are increasingly wary of farm-raised meat products owing to the perception of unacceptable use of additives required in their production. This applies to aquaculture products as it does to more conventional farmed products. And lastly, shorefront homeowners don't want buoys or cages that spoil their view of the waterway.

Perhaps a few shellfish and finfish species can be found that could be cultured in the estuaries in tiny selected areas for "niche" gourmet and ethnic markets. The shellfish species might be *A. irradians*, high quality *C. virginica*, heat tolerant *M. arenaria*, and cockles (*Anadara* sp.) and others. Such species need to be selected, growout areas found, and after-markets identified. Then hatchery-growout culture can be successful.

OYSTER PRODUCTION AT FISHERS ISLAND OYSTER FARM. Steve Malinowski, Fishers Island Oyster Farm, Fishers Island, NY 06390

Fishers Island Oyster Farm has been in operation for 22 years; first as a hard clam (*Mercenaria mercenaria*) producer and for the last 16 years as a producer of American oysters (*Crassostrea virginica*). During 2003, 650,000 oysters were produced exclusively for the half shell trade. Additionally, sales of seed oysters and hard clams account for approximately one third of total revenues.

Oyster and clam seed is produced in a 22' × 28' hatchery and transferred first to land based upwellers at 1 mm and then to FLUPSYs at 3–4 mm. After attaining 12 mm, oysters are stocked into pearl nets and grown for the remainder of the first season in a salt pond. The following spring, approximately 20% to 30% of the oysters are stocked into 5 tier lantern nets and suspended from long lines of buoys in West Harbor, Fishers Island Sound (5-acre New York State Temporary Land Use Assignment). The remainder of the seed is sold to other growers.

Due to fouling organisms and the habit of oysters growing into one another and the mesh of the nets, a considerable amount of handling is required. All work is accomplished with two motorized rafts (8' × 16') in the salt pond and two skiffs (21') in West Harbor. By the end of the second growing season (18 mo from spawning) approximately 50% to 75% of oysters are marketable.

SURVIVORSHIP OF WIND-DRIVEN BEACHED BAY SCALLOPS, *ARGOPECTEN IRRADIANS IRRADIANS*, AFTER RETURN TO NANTUCKET HARBOR. Andrew W. McCandless,^{1,2} Peter B. Boyce,³ Robert S. Kennedy,³ W. Forrest Kennedy,⁴ Valerie A. Hall,⁵ and Frank A. Dutra^{6,7}, ¹Maria Mitchell Association, 4 Vestal Street, Nantucket, MA 02554; ²Nantucket High School, 10 Surfside Road, Nantucket, MA 02554; ³Maria Mitchell Association, 4 Vestal Street, Nantucket, MA 02554; ⁴Department Of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853; ⁵Nantucket High School, 10 Surfside Road, Nantucket, MA 02554; ⁶Maria Mitchell Association, 4 Vestal Street, Nantucket, MA 02554; and ⁷Nantucket Shellfish Association, 147 Orange Street, Nantucket, MA 02554

On November 14 and 15, 2003 during high northwest winds and wind-driven higher tides than usual, large numbers of bay scallops, *Argopecten irradians irradians*, washed ashore at Wauwinet and Pocomo beaches in Nantucket Harbor. On November 15

and 16, scallopers and volunteers rescued hundreds of thousands of scallops from the shore, and deposited them back into the harbor. To determine the survivorship of the rescued scallops and thus the value of the scallop rescue effort, we placed 351 scallops in four modified wire lobster traps in 2 m of water (MLW) west of Pocomo Head in Nantucket Harbor on November 16. Group 1 contained 80 seed scallops exposed for 3 hours or less on the Pocomo shoreline; Group 2 and 3 contained 70 large seed and/or "nub" scallops each, recovered from the eelgrass wrack in Wauwinet that had been there for 12 hours or less; and Group 4 contained 131 seed exposed in the Wauwinet wrack from 24 to 36 hours. After 42 days on December 28, we found 340 live, and only 11 dead scallops in the pens: 1 dead in Group 1, 2 in Group 2, 6 in Group 3, and 2 in Group 4. This represents a 96.9% survival rate of the rescued scallops.

HOW HIGH PH'S CAN AFFECT THE CHEMISTRY IN LARGE VOLUME CULTURES OF *TETRASELMIS CHUI* (PLY429). Shannon L. Meseck and Barry Smith, USDOC, NOAA National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

For microalgal photosynthesis to occur, free dissolved carbon dioxide or carbon dioxide in the form of carbonate ion, needs to be present. In natural aquatic ecosystems, dissolved carbon dioxide generally is high enough to not limit phytoplankton growth. In high-volume mass cultures of microalgae (e.g., 18,000-L tanks), however, carbon dioxide may limit photosynthesis and growth, thereby decreasing the potential biomass of a culture. The amount of dissolved carbon in the system not only affects photosynthesis, but it can also affect the pH of the cultures. Nutrients are extremely sensitive to pH changes. For example, at a high pH, ammonia will exist predominately as unionized ammonia rather than ionized ammonium.

To investigate the chemical variability in large-volume cultures, 6 replicate 100-L kalwall tubes were inoculated with PLY429. Based upon the amount of nitrogen and phosphate added (f/2 commercial mix), all 6 algal cultures were expected to be nitrogen limited. Three cultures were bubbled with carbon dioxide so that the pH would be maintained below 9, whereas the other 3 did not receive any supplemental carbon dioxide, thus the pH could fluctuate. Cultures were incubated in a greenhouse during April of 2003. Approximately every 8 hours, samples were taken to determine dissolved inorganic carbon, alkalinity, pH, light, temperature, biomass, and particulate carbon and nitrogen. In tanks receiving carbon dioxide, final cell numbers were twice as high as in tanks that were not bubbled with carbon dioxide. The reduction in cell numbers for the tanks without carbon dioxide may be associated with low phosphate availability. In the tanks without carbon dioxide, dissolved phosphate levels became undetectable within 3 days; whereas, in the tanks with carbon dioxide, dissolved phosphate levels remained high. Phosphate can complex with other

chemicals in the media (i.e., calcium, magnesium, sodium, and iron) and render it less bioavailable for phytoplankton uptake. Phosphate complexing with these chemicals is dependent on their relative concentrations and on the pH of the media. Equilibrium calculations and our data suggest that in the cultures in which pH was not controlled (no carbon dioxide addition), phosphate may have reacted with other chemicals in the media (most likely calcium) to form a complex that was not available to the phytoplankton for growth. Thus, it seems that if pH is not controlled, resulting changes in the chemistry in large cultures of algae can influence algal growth.

SHELLFISH AQUACULTURE: THE CANARY IN THE MINESHAFT. Bill Mook, Mook Sea Farm, 321 State Route 129, Walpole, ME 04573

Mook Sea Farm has been in business for 19 years. We are located in mid-coast Maine on the Damariscotta River, where we have a 6,000 square foot hatchery and access to a little over 30 acres of oyster nursery and bottom culture leases.

In a typical year the hatchery produces more than 100 million 1 to 2 mm seed shellfish. Throughout the late 1980s and 1990s we experienced varying levels of success with our spawns, but were able to attain the desired levels of hatchery output by doing many large spawns, some of which did well enough to provide the necessary numbers of animals. We attributed the variable outcomes to the "vagaries of nature."

The "vagaries" began to get worse in 1997, but they didn't command our full attention until we started up the hatchery in 1998. Beginning with our first spawn of that year in late January, we were unable to bring a single hard clam spawn through metamorphosis. The quest for reasons began in February, and by early summer became essentially our sole focus. All of the hatchery systems were scrutinized; we hunted for a disease, and investigated the possibility of metal contamination caused by stray electrical current. By early September all lines of inquiry had led nowhere, half the company's employees had been let go, and Mook Sea Farm's future was in grave danger. Fortunately we got a tip-off: our neighbor on the river was dumping septic and porta-potty waste into the Damariscotta River near our hatchery intake.

Armed with this information we immediately started a surveillance effort. We documented the dumping, sampled the effluent and demonstrated that it produced the same effects on *Mercenaria* larvae that we had observed earlier in the year while attempting to produce seed. The ensuing regulatory and legal effort successfully shut down the illegal dumping and, beginning in 1999, Mook Sea Farm was able to produce shellfish in its hatchery again.

The experience caused us to carefully evaluate the company's goals. One of our new missions was to reach a high level of predictability in the hatchery. With good water quality and persistent effort we have achieved a high level of production efficiency

and a spawn success rate of 100% over the last 2 years. The "vagaries" seem to be safely locked in the closet.

LIPID ENRICHMENT OF EASTERN OYSTER BROOD-STOCK FOR INCREASED LARVAL SETTLEMENT. Gregg Rivara and R. Michael Patricio, Suffolk County Marine Environmental Learning Center, Cornell Cooperative Extension of Suffolk County, 3690 Cedar Beach Road, Southold, NY 11971

The link between certain fatty acids and the health of shellfish is well established. This project was designed to determine if a lipid emulsion could take the place of high lipid algal strains in the diet of oysters to maintain production while reducing the complexity and costs of operating a shellfish hatchery. Eastern oyster broodstock were fed a commercially available lipid supplement (Super Selco) designed for enriching the live feeds of finfish larvae. In one treatment, the supplement was fed in combination with T-Iso, the second feeding protocol was a high lipid strain of *Tetraselmis* and T-Iso, whereas the third was T-Iso alone (control). We measured how this supplement affected oyster broodstock conditioning in relation to fecundity, egg size, egg lipid content, and fertilization rate. In addition, we also examined late-feeding oyster larvae (before eyespot formation) as to how broodstock lipid supplementation affected set rate. Set rates of oysters fed Selco + T-Iso and *Tetraselmis* + T-Iso were higher than those fed T-Iso alone. The results of this work may be of interest to shellfish hatchery managers and remote setting operators.

This abstract is based on work supported by the Cooperative State Research, Education, and Extension Service (CSREES), US Department of Agriculture, under Agreement No. 98-38500-5917 awarded to the Northeastern Regional Aquaculture Center at the University of Massachusetts Dartmouth. Project Code 02-10.

IMPLEMENTING THE REMOTE SETTING TECHNIQUE FOR THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*, IN THE NORTH CAPE OIL SPILL SHELLFISH RESTORATION PROGRAM. Kate Ryan,¹ Karin A. Tammi,² and Michael A. Rice,³ ¹Department of Biologic Sciences, University of Rhode Island, Kingston RI 02881; ²RIDEM Division of Fish and Wildlife Coastal Fisheries Laboratory, Wakefield RI 02879; ³Dept. of Fisheries Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881.

The North Cape Shellfish Restoration Project is an attempt to repopulate the coastal ponds of Southern Rhode Island following the devastating effects of the *North Cape* Oil Spill in 1996. One major aspect of the restoration project is to repopulate the oysters in the coastal ponds. This is the first time an oyster remote set project of this size has been attempted in Rhode Island, and there are questions that need to be answered to make this project successful in the future. The focus of this study is to determine which type of locally available cultch material (surf clam, *Mactromeris*

solidissima or the ocean quahog, *Arctica islandica*) the oyster larvae prefer as a settlement substrate and to determine the growth rates of the post-set oysters in nursery trays. Remote setting of *Crassostrea virginica* larvae was carried out early on June 9, 2003 and approximately 1 million juvenile oysters on cultch materials were placed into nursery trays in Point Judith Pond on June 25. There was no statistical difference in oyster settlement onto the two substrate types. Between July 9 and October 29 mean heights of oysters on the cultch material in nursery trays increased from 5.0 to 34.0 mm, with greatest linear growth rates occurring the months of July and August. During early November 2003, oysters from the nursery trays were transported to four restoration sites in Rhode Island: Potters Cove, Spectacle Cove, Bissel Cove, and Pt. Judith Pond.

SOME IMPLICATIONS OF CONTROLLING CO₂ SUPPLY TO CULTURES OF *TETRAELMIS CHUI* (PLY429). Barry Smith and Shannon L. Meseck, USDOC, NOAA National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

The addition of carbon dioxide (CO₂) gas to marine algal cultures has been practiced by many for some time, but the quantity added is seldom matched closely to actual carbon demand. Carbon dioxide gas dissolves in seawater and dissociates to carbonic acid (CO₂+H₂O<=>H₂CO₃<=>H⁺+ HCO₃⁻). This causes a drop in pH, which can be reversed when dissolved carbon is consumed by algae during photosynthesis. Accordingly, CO₂ addition can be used to control the pH in an algal culture and to supply carbon as needed to the algae.

In April of 2003, six 100-L open cultures were started in The Greenhouse for Research on Algal Mass Production Systems (GRAMPS). The six cultures were grown in filtered seawater with Guillard's [f/2] nutrients added at time zero. Three of the cultures were not pH controlled (no CO₂ added). The other three cultures were maintained at a pH below 9.0 by daily CO₂ addition. Cultures were sampled periodically for measurements of pH, cell number, and macronutrients. The amount and time of CO₂ additions as well as weather conditions were also recorded.

Algal cultures that were controlled with CO₂ addition grew to a higher density faster than cultures that were not controlled. An average of about 1800 mL CO₂ was bubbled into each of the controlled cultures per day. The pH of uncontrolled cultures rose to over 10.0 by the 5th day. The detection of contaminants occurred at the same rate in all cultures. Further, there were no detectable differences in the blue-green algal contaminant.

The optimal amount of carbon addition should be related to the photosynthetic needs of the algae, with consideration of carbon cycling by respiration of both algae and bacteria in the culture. The optimal addition rate of carbon to the culture may be dictated by the magnitude of the resulting pH change and thus a possible physiologic shock to the algae. An automated pH monitoring and

control loop could be used to add enough carbon, in suitable doses, to satisfy algal needs and avoid large pH fluctuations. By optimizing both amount and rate of carbon addition, a control loop can be an economically desirable way to achieve higher cell densities, faster than in "traditional" static algal cultures.

A REVIEW OF GENETIC STUDIES ON COMMERCIAL SPECIES OF BIVALVES. Sheila Stiles, Joseph Choromanski, and Dorothy Jeffress, USDOC, NOAA National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

With significant advances made in the culture of marine organisms has come increased interest in improving genetic traits, especially of economic importance, such as growth, survival, and disease resistance for increased productivity. The overall goal of breeding or management of a species is to maximize productivity. An understanding of what it takes to maximize production enables the breeder or culturist to recognize signs of inbreeding depression which could be manifested as slower growth, decreased viability, disease susceptibility or overall decreased production. An example of inadequate attention to genetic consequences of culture with bay scallops occurred when some of the industry in Asia collapsed; which was attributed to inbreeding depression from a narrow gene pool.

Genetics of commercial oysters, clams, and scallops will be reviewed from such perspectives encompassing 3 aspects: breeding or quantitative genetics, chromosomal or cytogenetics, and molecular (DNA) genetics. These areas of genetics have been applied to commercial bivalves separately and in combination with varying degrees of success as measured by different responses for hatchery culture and in field programs. Conventional approaches consist of breeding methodology similar to that applied historically in agricultural genetics with the domestication of farm animals and crops. Heritability analyses of growth and selective breeding have provided positive responses in bivalves. Chromosome manipulation to induce polyploidy and cloning also has produced some favorable results. Alternatively, biotechnologic techniques can be used to facilitate progress in improvements. Various types of molecular markers are being used to supplement conventional approaches of breeding to improve characteristics with quantitative trait loci (QTLs) in marker-assisted selection (MAS). In addition, genetic markers are being used to identify stocks and to estimate genetic diversity of wild populations. DNA markers have been observed to result in variation with many alleles that could be useful for applications such as species, stock, and population identification. If environmental and habitat qualities are not suitable, however, genetic improvement in desired traits may not find ex-

pression, an important consideration for future developments in increasing the commercial production of bivalves.

THE ROLE OF DISEASE IN CONNECTICUT'S BIVALVE AQUACULTURE. Inke Sunila, State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97, Milford, CT 06460

There are 6 aquaculture bivalve species in Connecticut: Eastern oysters (*Crassostrea virginica*), quahogs (*Mercenaria mercenaria*), softshell clams (*Mya arenaria*), razor clams (*Ensis directus*), bay scallops (*Argopecten irradians irradians*), and blue mussels (*Mytilus edulis*). Several factors cause pathologic changes in bivalves: toxic algae, predation, fouling, salinity and temperature extremes, pollutants, hypoxia, old age, siltation, starvation, and infectious agents. Infectious agents in oysters include papovaviruses (viral gametocyte hypertrophy), Rickettsia, ciliates, *Perkinsus marinus* (Dermo), *Haplosporidium nelsoni* (MSX), *Haplosporidium costale* (SSO), *Nematopsis ostracorum*, trematodes and turbellaria. *P. marinus* and *H. nelsoni* infections are commercially important diseases. *P. marinus* prevalences were 60% to 80% between 1997 and 2002. However, the intensity of the infection (Mackin scale 1–5) remained around 1. Intensity of at least 3 is required to cause mortalities, so the high prevalence, low intensity *P. marinus* infection in oysters was not associated with significant mortalities. *H. nelsoni* caused significant mortalities in 1997–1998, but since then the epizootic prevalences have constantly decreased from the peak value of 47% in 1997 to 3% in 2002. Oyster production was low despite the low disease prevalences. Factors other than disease regulated oyster stocks in CT. Unlike southern estuaries, successful oyster sets in CT do not occur yearly, but several years apart. Commercial oyster sets occurred in 1969, 1973, 1987, and 1997. No sets have occurred since 1997 and commercial oyster inventory is low. The proportion of hatchery raised oysters in CT's production is presently only 4%. Quahog production is increasing and CT is the number one quahog producer on the East Coast. Infectious agents in quahogs include *Perkinsus* sp., *Chlamydia*, and QPX (Quahog Parasite Unknown, Labyrinthulomycota, Thaumochytridae). QPX, an economically important clam parasite, had a prevalence of 0.2% (3 out of 1764 clams studied in 1997–2003) and doesn't presently pose a threat to CT's quahog harvest. Infectious agents in softshell clams include *Perkinsus* sp. and the agent causing disseminated sarcoma, presumably a retrovirus. Infectious agents in bay scallops include trematodes and kidney coccidia. Infectious agents in blue mussels include *Chlamydia*, *Ancistrum mytili* ciliates, the microsporidian *Steinhilfsii mytilovum* (Mussel Egg Disease) and the trematode *Proctoeces maculatus*. *S. mytilovum*, and *P. maculatus* are economically important mussel parasites that pose a threat to potential CT mussel aquaculture. Although the list of infectious agents in CT's bivalves is extensive, parasite pressure alone doesn't restrict possible expansion of bivalve aquaculture. The most important biologic factor

currently posing a threat to the industry is the increasing occurrence of toxic algae blooms.

INDUCED TRIPLOIDY IN THE BAY SCALLOP, ARGOPECTEN IRRADIANS IRRADIANS, AND FIELD CULTURE PERFORMANCE. Amandine S. Surier and Richard C. Karney, Martha's Vineyard Shellfish Group, PO Box 1552, Oak Bluffs, MA 02557

Triploidy is the condition of possessing three times the haploid number of chromosomes in the cell nucleus. It can be induced by cell treatment, in the early stages of development. Not to be confused with controversial, transgenic genetically-modified organisms (GMOs), polyploidy is simply an increase in the number of a single organism's chromosomes, with no introduction of foreign genetic material. Triploid animals and plants are usually sterile because of the inability of homologous chromosomes to synapse in meiosis. As a result of their sterility, they tend to divert the energy usually used for reproduction towards somatic growth. Because of this unique quality, triploid bivalve production has attracted worldwide attention since the early 1980s. At present triploidy has been successfully applied to the economic benefit of the Pacific oyster industry on the west coast of the United States and also in France.

Triploidy in scallops has been investigated in several species over the past 20 years. Overall, research suggests that the development of a triploid bay scallop will result in an increased growth rate, a larger adductor muscle mass and an increased hardness with regard to stressful environmental conditions.

To test this hypothesis, triploidy was induced in the bay scallop *Argopecten irradians irradians*, under funding from the Northeastern Regional Aquaculture Center. The newly fertilized eggs were treated with a low risk chemical, 6-DMAP, that has been shown to be slightly less efficient than Cytochalasin B but much safer to handle as well as water soluble. The success of induction was measured by flow cytometry at the Virginia Institute of Marine Science. Triploidy was induced on five occasions during the 2003 culture season. Each batch of eggs was treated 15 to 25 minutes post fertilization for approximately 11 minutes with a treatment concentration of 400 μ M. The results ranged from 77% up to 97% triploidy in the first week of development. In late August and early September, juveniles from the last two spawns were deployed in growth trials in Katama Bay on Martha's Vineyard, MA, Buzzards Bay on Cape Cod, MA, in Point Judith Pond, RI, and in several bays on eastern Long Island. The shell growth was measured every 2 weeks at each site until the end of the growing season.

COMMUNAL EFFECTS BETWEEN ARGOPECTEN IRRADIANS IRRADIANS AND NASSARIUS OBSOLETUS. David J. Veilleux and Katlyn Mihalek, USDOC, NOAA National Marine Fisheries Service, USDOC, NOAA Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Groups of bay scallops, *Argopecten irradians irradians*, and mud dog whelks, *Nassarius obsoletus*, were placed in pearl nets to

test if the presence of the snails would control biofouling and reduce sedimentation within the suspended culture gear. The nets were hung for 33 days to attain settlement of the fouling communities and the level of sediments common to aquaculture gear. We compared survival and growth of scallops in treatments ($n = 2$) with and without the presence of snails. The wet weights and shell heights were measured for all animals at the start and finish of the experiment. Scallop wet weight averaged 32 g greater in treatments containing snails compared with those without snails. This increase in weight may be attributed to the snail's ability to clear the mesh of fouling, therefore increasing water flow rates to the scallops. The results from this trial suggest that this simple low-cost approach may improve aquaculture productivity.

GROWTH ENHANCEMENT OF DISEASE-RESISTANT STRAINS OF THE EASTERN OYSTER THROUGH TRIPLOIDY. Yongping Wang, Ximing Guo, Gregory DeBrosse and Susan Ford, Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349

The eastern oyster (*Crassostrea virginica*) faces three major diseases: MSX (caused by the parasite *Haplosporidium nelsoni*), Dermo (caused by the parasite *Perkinsus marinus*), and the juvenile oyster disease (JOD, probably caused by a bacterium). Rutgers University has been breeding oysters for disease-resistance since the early 1960s. Strains resulting from the Rutgers breeding program have shown strong resistance to MSX and recently some resistance to Dermo. Whereas the Rutgers strains are superior in disease-resistance, they do not grow as fast as some of the commercial strains, such as the Frank M. Flower Oyster Company's strain. Growth improvement of the Rutgers disease-resistant strains is needed to maximize return for oyster farmers. Triploids grow significantly faster than diploids in most mollusks studied so far. We recently developed tetraploid oysters from the Rutgers disease-resistant strains. The goal of this study is to produce mated triploids from the disease-resistant tetraploid stocks and compare their growth and survival to that of normal diploids and chemically induced triploids.

Three replicates of 3 groups were produced from Rutgers disease-resistant stocks: (1) diploid control (2N); (2) triploids induced by blocking polar body II with cytochalasin B (3nCB); and (3) natural triploids produced by diploid female \times tetraploid male crosses (3nDT). The 3nCB groups produced 72% triploids on average, and the 3nDT groups produced 98% triploids. Oysters were measured and individually assayed for ploidy confirmation. At 17 months of age, oyster size was in the general order of 3nDT > 2n > 3nCB. Triploids from 3nDT were significantly bigger than 2N diploids and 3nCB triploids in shell height, and significantly bigger than 2N diploids in whole body weight. Preliminary results from this study suggest that mated triploids may provide significant growth enhancement over diploids and chemically induced triploids.

EFFECTS OF 96-HOUR EXPOSURE TO AMMONIA ON BAY SCALLOPS, *ARGOPECTEN IRRADIANS IRRADIANS*. James C. Widman Jr., Shannon L. Meseck, George Sennfelder and David J. Veilleux, USDOC, NOAA National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

In either static, semi-static, or recirculating culture systems nitrogenous waste products are produced by bay scallops, *Argopecten irradians irradians*, during their normal metabolic activities. The toxic components of these waste products need to be controlled or converted to less lethal forms or they will result in scallop mortalities. Little research has been done to determine the lethal concentrations of un-ionized ammonia in juvenile bay scallops (<30 mm). The ratio of ammonia in the ionized and un-ionized form (with the latter being the toxic form) is dependent on pH, temperature, and salinity.

In this experiment, salinity and temperature remained constant while pH (7.6, 8.0, and 8.4) and ammonia levels (0, 6, 12, and 18 mg N L⁻¹) were varied resulting in a 3 \times 4 grid. Scallops were not fed during the 96-hour exposure to ammonia to prevent uptake by phytoplankton. Un-ionized ammonia concentrations ranged from 0 mg to 5 mg L⁻¹. At un-ionized ammonia concentrations greater than 0.60 mg L⁻¹ we observed 100% bay scallop mortality. Un-ionized ammonia concentrations of approximately 0.45 mg L⁻¹ resulted in 50% scallop mortality. At a pH of 8.4 total ammonia concentrations as low as 6 mg L⁻¹ resulted in 100% scallop mortality. Monitoring of ammonia and pH is vital to maintaining high survival in static and recirculating scallop rearing systems.

DETERMINING OPTIMUM FEEDING CONCENTRATIONS FOR BAY SCALLOPS, *ARGOPECTEN IRRADIANS IRRADIANS*, USING AN AUTOMATED CONTROL SYSTEM. James C. Widman Jr. and David J. Veilleux, USDOC, NOAA National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460

Technologic changes over the last 30 years allow us to make new observations into the filter feeding behavior of bay scallops, *Argopecten irradians irradians*. We have developed an automated feeding system that uses a fluorometer to monitor the fluorescence of phytoplankton suspended in culture water as an indication of cell concentration. Fluorometer readings are acquired by a personal computer and input to an algorithm that can: (1) terminate the addition of algae; (2) start the addition of algae; and (3) continue at the present feeding status. This system allows us to quan-

tify the frequency and duration of feeding in a semi-static closed culture system.

A series of range finding experiments were conducted with bay scallops to explore optimum feeding concentrations and growth. All experiments were conducted at 24°C using 10 µm filtered seawater. During trial one, scallops were grown in seawater with *Tetraselmis chui* (PLY429) concentrations of 15,000, 25,000, 35,000, and 45,000 cells/mL. Scallops ranging in initial mean shell heights of 6.7–7.1 mm grew to 9.2–9.9 mm in 28 days with no significant difference ($P > 0.05$) in growth. In the second trial, scallops were grown in seawater with *Tetraselmis chui* PLY429 concentrations of 5,000, 10,000, 15,000, and 25,000 cells/mL. Scallops ranging in initial mean shell heights of 6.6–7.1 mm grew to 12.8–13.2 mm in 23 days with no significant difference ($P > 0.05$) in growth. In the third trial, scallops were grown in seawater with *Tetraselmis chui* (PLY429) concentrations of 1,000, 2,333, 3,667, and 5,000 cells/mL. Scallops ranging in initial mean shell heights of 5.8–6.2 mm grew to 13.6–14.8 mm in 36 days. We found a significant difference ($P < 0.05$) in growth of bay scallops held at these concentrations. Scallops grown at 2,333 cells/mL were similar to those grown at 1000 cells/mL and significantly larger than those grown at higher cell concentrations.

These preliminary results indicate that growing scallops at low phytoplankton concentrations yields faster growth. Scallop growth in our experiments was optimized at 2,333 cells/mL, but further studies will refine the relationship between cell concentration and optimal growth.

THE ESTUARINE DINOFLAGELLATE, *PROROCENTRUM MINIMUM*—“HAB” OR “HAB-NOT?” Gary H. Wikfors,¹ Jennifer H. Alix,¹ Roxanna M. Smolowitz² and Hélène Hégaret³, USDOC, NOAA ¹National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; ²Marine Biological Laboratory, Woods Hole, MA 02543; ³Department of Marine Sciences, University of Connecticut, Groton, CT 06340

Field observations and laboratory studies showing a variety of harmful effects of *Prorocentrum minimum* upon molluscan shellfish have not resulted in a consensus on whether *P. minimum* “is toxic” to grazing molluscs. Mortalities of marine life coincident with natural blooms of *P. minimum* sometimes seem to be associated with resultant hypoxia, but not always. Laboratory experiments testing the response of grazing molluscs to *P. minimum* cultures have yielded variable results, ranging from mortality to normal growth, and including rejection as pseudofeces, poor development, tissue pathologies (sometimes transient), and systemic immune responses. The variability in trophic interactions between *P. minimum* and molluscan shellfish suggests variation in the expression of “toxicity” in this species, although a specific chemical compound has not been identified as “the toxin.” Several recent studies have provided strong evidence that variation in toxicity is dependent upon environmental conditions and their effects upon the physiology of *P. minimum*. Accordingly, seemingly conflicting observations from field and laboratory studies may be explained by the transient nature of toxin expression in this dinoflagellate.

ABSTRACTS OF TECHNICAL PAPERS

Presented at the 57th Annual Meeting

NATIONAL SHELLFISHERIES ASSOCIATION

(Pacific Coast Section)

&

PACIFIC COAST SHELLFISH GROWERS ASSOCIATION

Portland, Oregon

October 8–11, 2003

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ASSESSING THE IMPACTS OF URBANIZATION ON SHELLFISH GROWING AREAS IN PUGET SOUND.

Marina Alberti, Department of Urban Design and Planning, University of Washington, Box 355740, Seattle, WA 98195; **Marcie Demmy Bidwell**, Department of Landscape Architecture, University of Washington, Box 355740, Seattle, WA 98195; and **Stuart Glasoe**, Puget Sound Action Team, Office of the Governor, PO Box 40900, Olympia, WA 98504-0900.

Shellfish are prized icons of the Pacific Northwest, associated with many recreational, cultural, and economic values. Clean water is essential for shellfish harvesting. However, an increase in human population and development within near-shore environments and adjacent watersheds has negatively impacted water quality, resulting in increased closures of fishing, contact recreation, and shellfish harvesting. Though research has shown that urbanization alters water quality through changes in reduced native vegetative cover, increased impervious surfaces, altered hydrology, and other impacts, the relationships between patterns of urbanization and shellfish growing areas are poorly understood. We developed an empirical analysis of 32 basins in Puget Sound with variable degrees of urbanization to test the formal hypotheses regarding the impact of urban patterns on shellfish growing areas. The sites were selected to represent a gradient of urban land use/land cover patterns. Our working hypothesis is that variations in land cover composition, landscape configuration, and connectivity of impervious areas explain most of the variation in near-shore water quality conditions. Using bacterial contamination as the indicator of near-shore conditions, we develop a comparative or cross-sectional analysis across the 32 basins to assess what landscape factors best explain water quality conditions in Puget Sound's shellfish growing areas. We discuss the implications of our results and recommendations to policymakers and resource managers within the Puget Sound region.

RECENT MORTALITY EVENT OF PACIFIC RAZOR CLAMS, *SILIQUA PATULA*, ALONG THE PACIFIC COAST OF WASHINGTON STATE ASSOCIATED WITH RECORD INFECTION INTENSITY OF THE GILLS BY NUCLEAR INCLUSION X (NIX).

Dan L. Ayres, Washington Department of Fish and Wildlife, Montesano, WA 98563; **Ervin J. Schumaker**, Quinalt Department of Natural Resources, PO Box 189, Taholah, WA 98587; and **Ralph Elston**, Aquatechnics, PO Box 687, Carlsborg, WA 98324.

After the summer 2002 razor clam stock assessment, a record number of adult razor clams were determined to be available for harvest on the five management beaches located along the Washington State coast. However, due to high levels of the marine toxin domoic acid, no harvest occurred on any management beach with the exception of a limited tribal harvest on the northern beaches before the toxin arrived. Coincidentally, a sample of 50 clams was collected in early October for analysis of infection intensity of

nuclear inclusion X (NIX). NIX is a primitive, large bacterium obligately infecting the nucleus of gill epithelial cells of the razor clam. NIX is a member of the gamma subclass of the eubacterial class Proteobacteria. NIX was the suspected cause of a razor clam mortality event in 1983 that resulted in the loss of more than 90% of the adult clams found on some management beaches as well as high percentage losses in 1985 and 1989 associated with high NIX infection intensities. The high NIX infection intensities are followed by ruptured gill epithelia that may be synchronized by an environmental temperature drop. In October 2002, we found an average NIX infection intensity of 48.3, the highest ever recorded since records were first made in 1983. The summer 2003 razor clam stock assessment found a 49.7% loss of adult clams coast-wide. NIX is again the likely suspect.

STATUS OF THE EUROPEAN GREEN CRAB INVASION.

Sylvia Behrens Yamada and **Marsha Becklund**, Zoology Department, Oregon State University, Corvallis, OR 97331.

The recent invasion of Pacific Northwest estuaries by the European green crab, *Carcinus maenas*, caused much initial alarm. Following the strong El Niño of 1997–1998, young green crabs appeared in estuaries along the coasts of Oregon, Washington, and as far north as Nootka Sound on the west coast of Vancouver Island, British Columbia. Unusually strong northward-moving coastal currents of up to 50 km/day must have transported larvae from more established populations in California to the Northwest. Coastal transport events have been much weaker in recent years. Crabs from the 1997–1998 colonizing year class are still in the population. Males now range from 86 to 98 mm in carapace width. We predict that most of the colonizing year class will die of senescence by the end of 2004. Though we have trapped young green crabs (less than 50 mm in carapace width) in 1999–2003, their abundance has been at least one order of magnitude lower than in 1998. Lack of larval supply appears to prevent green crabs from expanding their range at this time. We predict that favorable ocean conditions, associated with strong El Niño events, will result in an increase in green crab abundance and range expansion. Even though green crab abundance in the Northwest is low when compared with Europe, eastern North America, and California, it is imperative to continue monitoring efforts to elucidate the process of range expansion of non-indigenous marine species and to serve as an early warning system for the next strong recruitment event of green crabs.

DEVELOPMENT AND IMPLEMENTATION OF AN IPM PROGRAM FOR BURROWING SHRIMP IN WILLAPA BAY/GRAYS HARBOUR, WASHINGTON.

Steven Booth, Willapa Bay-Grays Harbor Oyster Growers Association, Nahcotta, WA 98637; and **Bill Dewey**, Taylor Shellfish, Shelton, WA 98584.

In March 2003, a "Comprehensive plan towards an integrated pest management program for burrowing shrimp on commercial oyster beds" was submitted to the Washington Department of

Ecology. The plan was comprehensive as it included descriptions of relevant concepts, definitions and goals, references, lists of principal authorities and policies, and five interconnected key IPM elements: (1) funding, (2) research & development, (3) implementation, (4) evaluation/regulatory compliance, and (5) dissemination. Yet the plan remains incomplete within each element. It remains "a living document" as directed in the original Memorandum of Agreement between the Growers Association and several state agencies that mandated its development in 2001. Here, we present the recent (post-2001) history of the IPM plan for burrowing shrimp and its potential future(s) within the web of environmental, political, and agricultural concerns. The plan itself is described with special consideration given to how well it corresponds to the traditional IPM paradigm adopted for terrestrial agroecosystems.

BIOCHEMICAL AND HISTOLOGIC CHANGES IN RED ABALONE (*HALIOTIS RUFESCENS*) SUBJECTED TO DIFFERENT COMBINATIONS OF TEMPERATURE, FOOD AVAILABILITY, AND INFECTION WITH THE AGENT OF WITHERING SYNDROME. **Beverly A. Braid, James D. Moore, Thea T. Robbins, Ronald P. Hedrick,** School of Veterinary Medicine, University of California, Davis/Bodega Marine Laboratory, Bodega Bay, CA 94923; **Ronald S. Tjeerdema,** Department of Environmental Toxicology, University of California, Davis, CA 95616; and **Carolyn S. Friedman,** School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195.

We conducted a long-term study to identify pathophysiologic changes that occur during progression of withering syndrome (WS) and to contrast these with changes due to starvation in uninfected animals. Following antibiotic treatment to assure all animals were free of the bacterial agent of WS (WS-RLP), farmed red abalone were subjected to eight separate treatment combinations of exposure to WS-RLP (exposed or unexposed), temperature (ambient or 19°C), and food availability (fed or starved). The digestive gland (DG) retained up to 186 ppm oxytetracycline at day 105 post-treatment. Though 0.1% of abalone sampled from exposed/ambient groups became infected over 497 days, 56% of abalone sampled from exposed/19°C groups became infected. Metaplastic DG changes occurred only in infected abalone. Metaplastic DG did not exhibit proliferation or apoptosis differing from normal DG. Abalone in the exposed/fed/19°C treatment had significantly more DG changes and infection intensities than those in the exposed/starved/19°C group ($P < 0.05$). Foot glycogen decrease preceded shrinkage, foot degeneration, and decrease in condition index. Progression of WS shared many features with starvation although changes in DG architecture appear pathognomic for this disease. Supported by California Sea Grant College Program award R/A 117 and the Marine Region, California Department of Fish and Game.

THE EFFECTS OF LARVAL DISPERSAL ON GENETIC STRUCTURE OF BLACK ABALONES (*HALIOTIS CRACHERODII*). **Melinda D. Chambers, Carolyn Friedman, Lorenz Hauser,** School of Aquatic and Fisheries Sciences, University of Washington, Seattle, WA 98195; and **Glenn R. VanBlaricom,** Washington Cooperative Fish and Wildlife Research Unit, School of Aquatic and Fisheries Sciences, University of Washington, Seattle, WA, 98195.

Populations of black abalone have experienced declines of 85–99% throughout their range since the emergence of the bacterial pathogen causing Withering syndrome (WS) in 1985. Recovery of the species throughout its original range will depend on the ability for the remnant population to recruit successfully. The recruitment of organisms with a pelagic larval stage depends on oceanic physics coupled with larval characteristics of the species. It is thought that a high rate of larval dispersal could reduce the survival rate of a species recovering from depleted densities due to predation, starvation, or transport away from suitable habitat. A drift card study of sea surface circulation and a population genetics assessment was conducted to identify recruitment potential of black abalones throughout the California offshore islands. Drift cards were released from San Nicolas Island, California, in August 2002 and June 2003 corresponding with the two peaks in the spawning season of the species. Results indicate that free-floating surface particles are locally retained. To identify population structure of the species resulting from the dispersal of larvae, tissue was collected for genetic analysis from abalones on Santa Cruz, Santa Rosa, San Miguel, and San Nicolas Islands as well as from Vandenberg and Monterey on the California mainland. Results indicate that isolated populations are not significantly genetically differentiated but that genetic distance corresponds with geographic distance. Additionally, populations of recruit-sized individuals are more highly structured than adults at the same locations.

FOULING ORGANISM (*TRITICELLA* SP.) OF THE SPOT SHRIMP *PANDALUS PLATYCEROS*. **Richard K. Childers,** Washington Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, Brinnon, WA 98320; **Ralph A. Elston,** Aquatechnics, Sequim, WA 98382; and **Judy Winston,** Virginia Museum of Natural History, Martinsville, VA 24122.

A *Triticella* sp. (Bryozoa) was first observed as a fouling organism on the carapace of spot shrimp *Pandalus platyceros* in Hood Canal Washington in the fall of 2002. The prevalence and geographic distribution of the parasite on spot shrimp were studied and prevalence was analyzed by size, sex, and location of the shrimp. A total of 1931 shrimp were examined from twelve locations in Puget Sound. Prevalence of the parasite was high in shrimp from most locations sampled, ranging from a low of 0% (Port Townsend Bay) to 89% (Quilcene Bay), with an overall prevalence of 66%. The high prevalence of the fouling organism was associ-

ated with displacement of shrimp from their normal deep water habitat to shallow water during a period of low dissolved oxygen in Hood Canal, Washington. Large numbers of the bryozoan colonies were anchored to the carapace of the shrimp, particularly at articulations of carapace plates but also to appendages, except antennae. The colonies consisted of tufts of dense white stolons and zooids with spots of yellow brown coloration representing ingested algal cells. The bryozoans were not found attached to any soft tissues, but the sheer mass of attached organisms may have interfered with normal respiration and movement of the shrimp. Eleven species of the genus *Triticella*, typically epizootic on decapod crustaceans, have been named. Those found on the nektonic spot shrimp appear to represent an undescribed species, based on length and width of the zooids and stolon.

PREVALENCE, GEOGRAPHIC DISTRIBUTION, AND BIOLOGY OF A DUNGENESS CRAB, *CANCER MAGISTER*, MICROSPORIDIAN PARASITE. Richard K. Childers, Washington Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, Brinnon, WA 98320; Paul W. Reno and Robert E. Olson, Oregon State University, Hatfield Marine Science Center, Newport, OR 97356.

The prevalence and geographic distribution of *Nadelspora canceri*, a microsporidian parasite of the Dungeness crab *Cancer magister*, was studied and prevalences were analyzed by sex and size of host and date of collection. *Nadelspora canceri* was widely distributed along the U.S. Pacific Coast in estuaries from Bodega Bay, California, to southern Washington. Prevalence of the parasite was high in crabs from most estuaries sampled between 1991 and 1994, ranging from a low of 0.4% (Gray's Harbor, Washington) to 41.2% (Tillamook Bay, Oregon) with an overall mean of 14%. Crab examined from Grays Harbor in 2002 indicate the prevalence rate had increased from 0.4% to greater than 4%. The parasite also occurred offshore of California and Oregon, but was not found in Puget Sound, Washington, or in Glacier Bay, Alaska. The prevalence of infection in crabs captured in the open ocean was low (0.3%). In crabs collected near-shore at estuary mouths, prevalence was intermediate between estuarine and open-ocean levels. The infection was most prevalent in 2-y-old crabs (13-cm carapace width), and males had 2.5 times the rate of infection than did females. The mortality of laboratory-held crabs naturally infected with *N. canceri* was compared with that of uninfected crabs, and significantly higher mortality was observed for infected crabs. *Nadelspora canceri* infections were established in juvenile and adult crabs that were fed parasite spores in laboratory experiments indicating that transmission is direct and intermediate hosts or vectors are not required for transmitting the parasite.

THE RATE OF PREY CONSUMPTION IN TWO ESTUARINE CRAB SPECIES: THE INTRODUCED EUROPEAN GREEN CRAB, *CARCINUS MAENAS*, AND NATIVE DUNGENESS CRAB, *CANCER MAGISTER*. Timothy M. Davidson and Sylvia Behrens Yamada, Environmental Science Department and Zoology Department, Oregon State University, Corvallis, OR 97331-2914.

The European green crab (*Carcinus maenas*) is a voracious predator responsible for declines in numerous bivalve populations around the world. To predict the relative impact of *C. maenas* on bivalve prey in the Pacific Northwest, we compared prey consumption rates, claw morphology, and claw strength of *C. maenas* and the native Dungeness crab (*Cancer magister*). Field trials were conducted in a tidal channel from September 2, 2002, to September 14, 2002. Similar sized crabs were individually caged in sealed minnow traps and offered 50 mussels (*Mytilus trossulus*) (25–35 mm) per trial. Trials ranged from 48 to 72 h. After each trial, the numbers and biomass of consumed and partially consumed mussels were recorded. Both *C. maenas* and similar sized *C. magister* ate around 15 g of mussel meat per day. Though *C. maenas* and *C. magister* exhibit similar consumption rates, differences in claw morphology may allow the introduced crab to have a stronger effect on bivalve populations. *C. maenas* possess several superior claw characteristics including dimorphism, higher mechanical advantage of the claw lever system, and significantly larger propal heights in the master claw. These characteristics suggest that *C. maenas* has greater claw strength than *C. magister* and thus the ability to exploit larger and thicker shelled bivalves.

IMPLEMENTING A MONITORING PROGRAM FOR BURROWING SHRIMP AS PART OF AN INTEGRATED PEST MANAGEMENT PROGRAM IN WILLAPA BAY AND GRAYS HARBOR, WASHINGTON. Brett Dumbauld, Washington State Department of Fish and Wildlife, Willapa Bay Field Station, Ocean Park, WA 98640; D. Cheney, A. Suhrbier, H. Beltran, Pacific Shellfish Institute, Olympia, WA 98195; S. Booth and D. Tufts, Willapa Bay-Grays Harbor Oystergrowers Association, Nahcotta, WA 98637.

Two species of burrowing thalassinid shrimp cause significant problems for oyster aquaculture in West Coast estuaries by undermining the substrate that oysters grow on. An annual control program that uses the pesticide carbaryl applied directly to the estuarine mudflat has taken place in Washington State since 1963. The pesticide application program continues to be controversial, and the oystergrowers signed a memorandum of agreement with several state agencies and others in 2001 that requires that integrated pest management be pursued. In a recent out-of-court settlement, the growers also agreed to reduce and eventually stop carbaryl use. We designed and implemented a monitoring plan for burrowing shrimp in Willapa Bay and Grays Harbor, Washington, during 2002–2003. Monitoring plans are an integral part of any pest man-

agement plan, but monitoring shrimp populations in an estuarine setting is particularly challenging. Shrimp burrow deeply in the sediment, and pest abundance must be assessed by counting highly variable burrow openings that are difficult to distinguish from those of other organisms. Shrimp also display highly variable interannual recruitment patterns and oysters are planted on a two- to four-year schedule and therefore are akin to a perennial terrestrial crop. The data we collected and present suggest that monitoring is beneficial and that it may reduce costs and better target pest control efforts, regardless of the control tactic being used.

STATUS, PREVENTION AND MANAGEMENT OF DENMAN ISLAND DISEASE IN WASHINGTON STATE. Ralph Elston, Aquatechnics and Pacific Shellfish Institute, PO Box 687, Carlsborg, WA 98324.

Denman Island disease, caused by the protistan parasite, *Mikrocytos mackini*, was found for the first time in Washington State in May 2002. Surveillance continuing into 2003 confirms the infection of Pacific oysters in seven locations. The disease is considered to have no significance for Pacific oyster farming in British Columbia in most cases, with the exception of a few locations where three-year-old oysters are affected. Findings in Washington have been at low prevalence and often without visible pustular lesions, usually in larger feral oysters. The disease is known to be expressed at cooler water temperatures (ca. 10°C). Other species of oysters are susceptible to infection. The disease has significance to exporters of live shellfish products because it is considered a notifiable disease by the Office Internationale Epizooties (OIE). Most export producers have established a history of health surveillance and certification showing absence of the disease in brood and seed stocks. In conjunction with Canadian collaborators, we are conducting a risk analysis and risk management process and will recommend whether the disease should be listed as a notifiable disease to the OIE. Though there are some risks from this disease, other oyster diseases, both notifiable and not notifiable to the OIE, have clearly greater health consequences to the known host oyster. The primary risk to Pacific oysters may be in colder climates, although Washington seed oyster producers have been supplying oyster seed to Alaskan shellfish farms and there have been no reports of the disease in that state.

PROGRESS IN THE DEVELOPMENT OF EFFECTIVE PROBIOTIC BACTERIA FOR BIVALVE SHELLFISH HATCHERIES AND NURSERIES. Ralph Elston, Karen Humphrey, Aquatechnics, PO Box 687, Carlsborg, WA 98324; Arthur Gee, Daniel Cheney and Jonathan Davis, Pacific Shellfish Institute, Olympia, WA 98501.

We tested the efficacy of multiple probiotic bacterial candidates to protect Pacific oyster (*Crassostrea gigas*) larvae and juveniles against challenge by bacterial pathogens and to increase

survival of unchallenged hatchery-produced larvae. Probiotic bacterial strains were local isolates. In 12 replicated well plates and in scaled-up 1-L cultures of oyster larvae challenged with *Vibrio tubiashii*, with and without probiotic candidate P02-45 added at a concentration of 10^5 cfu per mL, survival of larvae was typically 40–80% greater in cultures with probiotic added. Addition of probiotic P02-45 to unchallenged hatchery production batches of larvae resulted in a survival improvement as great as from 21.0% without probiotic to 76.5% with probiotic. *In vitro*, both killed bacteria and cell-free extract were effective pathogen inhibitors. Compatibility of probiotic candidates P02-45 and P02-1 with seven species of algal food was determined. Probiotic candidates were compatible with some algal foods and, in some cases, resulted in an increased density of algal cells. Two compatible species were Tahitian *Isochrysis* sp. and *Rhodomonas* sp. Addition of algal cultures containing probiotic bacteria provided protection for larvae challenged with *Vibrio tubiashii* and a new filamentous bacterial pathogen. The probiotic was safe for oyster and other species of shellfish larvae up to a concentration of 10^7 cfu/mL. Above this concentration, the larvae appeared to die of anoxia due to the high bacterial concentration. Research supported by grant project number 2002-00362 from the U.S. Department of Agriculture, C.S.R.E.E.S., Small Business Innovation Research program.

THE EFFECTS OF NURSERY ENVIRONMENT ON THE PERFORMANCE OF ADULT PACIFIC OYSTERS *CRASSOSTREA GIGAS*. Ford Evans and Chris Langdon, Coastal Oregon Marine Experiment Station, Hatfield Marine Science Center, Department of Fisheries and Wildlife, Oregon State University, Newport, OR 97365.

A study was conducted to determine whether the rank-order of average family performance at harvest (2+ years old) was significantly altered by the feeding regimen that they experienced in the nursery as spat. Five full-sib oyster families were created and reared three different feeding regimes during the nursery phase. Forty individuals from each family-nursery treatment combination were stocked into each of 10 replicate lantern net tiers and deployed subtidally in Yaquina Bay, Oregon. Measurements of yield, individual weight, and survival were recorded after one and two growing seasons. Average family yield after two growing seasons was significantly affected by genotype ($P < 0.01$) but not by nursery environment ($P = 0.10$) or genotype \times nursery environment interaction ($P = 0.89$). Components of yield (i.e., individual growth rate and survival) were affected by both genotype and nursery environment ($P < 0.01$) but not genotype \times nursery environment interaction ($P > 0.82$). These results suggest that variation in nursery environment, manipulated by feeding regimen, does not significantly alter the rank-order of average family yield, growth rate, and survival evaluated after two growing seasons in the field.

BIOLOGY AND FISHERIES OF THE EXOTIC VARNISH CLAM (*NUTTALLIA OBSCURATA*) IN BRITISH COLUMBIA. Graham E. Gillespie and Neil F. Bourne, Fisheries and Oceans Canada, Pacific Biologic Station, Nanaimo, BC, Canada V9T 6N7.

Varnish clams, *Nuttallia obscurata*, have recently become established in southern British Columbia. They continue to expand beyond the Strait of Georgia into Johnstone Strait to Port Hardy and northwards along the west coast of Vancouver Island to Brooks Peninsula. They will likely become established in the Central Coast, although taking a different route than Manila clams. Experiments to examine competitive relationships between Manila and varnish clams showed evidence of competition when the two were placed together, with varnish clams having some advantage in the upper intertidal and Manilas in the mid-intertidal zone. Histologic examination revealed one spawning event per year, in July and August. Gonadal development started in January, and most clams were partially spent or inactive after September. Some clams in their second summer (~15–17 mm TL) showed evidence of early development of gonadal tissue, but most active spawners were greater than 25-mm TL, indicating that effective spawning commences two years after settlement. Varnish clams >30-mm TL were harvested from mixed populations, with harvest efficiency ~60–80% for this size class. Breakage during harvest was ~2%, and shrinkage during processing was ~4%, evenly divided between mortality and weight decrease due to water loss. Grit was purged readily within 48 h of wet storage, but commensal pea crabs were not purged after 34 days. Varnish clams have been added to aquaculture permits in BC, and most commercial harvest comes from leases. Commercial fisheries have not yet been successful.

THE EXPANSION OF AN INVASIVE CORDGRASS (*SPARTINA ALTERNIFLORA*) RESULTS IN THE LOSS OF CRITICAL FORAGING HABITAT FOR DUNGENESS CRAB (*CANCER MAGISTER*) IN WILLAPA BAY, WASHINGTON. Kirsten K. Holsman and P. Sean McDonald, School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195.

Tidally driven migrations are an integral component of the life histories of many brachyuran species, allowing crabs to use productive intertidal habitats at high tide and subtidal refuges at low tide. Results of a bioenergetic model for crabs in Willapa Bay, Washington, indicated that intertidal foraging is an essential element of the estuarine life history of *Cancer magister*, contributing significantly to the energy budget of subadult crabs (40–130 mm; 1+ and >1+ year classes), and likely facilitating the high abundances observed in large coastal estuaries. Acoustic telemetry studies and underwater video observations have shown that subadult *C. magister*, which reach densities as high as 4300 crabs ha⁻¹ in subtidal channels during low tides, regularly migrate during flood tides into intertidal habitats to forage. Furthermore, these

studies, as well as results of baited trapping surveys, revealed that subadult crabs are most abundant in unvegetated open sand or mud habitats at high tide. However, these habitats are increasing colonized by the invasive cordgrass *Spartina alterniflora*. Trapping surveys have shown that subadult crabs do not use *S. alterniflora* beds, and the rapid conversion of critical foraging habitats into inaccessible *S. alterniflora* patches threatens to significantly reduce crab populations within Willapa Bay. Because estuarine production of *C. magister* is a major contributor to the coastal population of adult crab, *S. alterniflora* expansion could potentially impact the coastal fishery.

THE EFFECT OF *ZOSTERA MARINA* AND *CRASSOSTREA GIGAS* CULTURE ON INTERTIDAL COMMUNITIES IN A NORTHEAST PACIFIC ESTUARY. Geoff Hosack, David Armstrong, School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195; Brett Dumbauld, Washington State Department of Fish and Wildlife, Willapa Bay Field Station, Ocean Park, WA 98640; Jennifer Ruesink, Brice Semmens, Department of Zoology, University of Washington, Seattle, WA 98195; and Ian Fleming, Department of Fisheries and Wildlife, Oregon State University, Newport, OR 97365.

Intertidal habitat structure created by seagrass and oysters is broadly considered to play an important role in the shaping of many biologic communities. In Pacific Northwest estuaries, commercial oysters are often cultivated at the same tidal elevation as seagrass meadows, and thus management decisions concerning resource use require insight into their comparative value. We compared the use of three important habitats by fish and crab within the low intertidal zone of Willapa Bay, Washington: (1) seagrass meadow (*Zostera marina*), (2) unvegetated tideflat, and (3) commercial oyster ground culture (*Crassostrea gigas*). We also sampled epibenthic meiofauna as a potential source of prey for fish and crab. Using passive gear (hoop nets) in 2001, we found no relationship between total CPUE or composition of nekton. Concurrent sampling with an epibenthic pump, however, showed that habitat has a strong effect on meiofaunal communities. Meiofauna densities were more than seven times higher in structured habitats, and composition was significantly affected by habitat. In 2002 and 2003, we focused on outmigrating juvenile chinook, chum, and coho salmon (*Oncorhynchus tshawytscha*, *Oncorhynchus keta*, and *Oncorhynchus kisutch*) using a modified tow net in the above habitat types plus adjacent subtidal channels. There was no evidence of a habitat effect, although region (within estuary) explained a significant amount of variation for each juvenile salmonid species.

EFFORTS OF TAYLOR SHELLFISH COMPANY TO ALLEVIATE THE PROBLEMS OF BURROWING SHRIMP ON AQUACULTURE GROUND IN WILLAPA BAY, WASHINGTON. Kurt Johnson, Taylor Shellfish Company, Shelton, WA 98584.

Oyster growers in Willapa Bay must find alternatives to controlling burrowing shrimp pests by spraying the insecticide Carbaryl. As part of this effort, Taylor Shellfish is testing several ideas. The approach is to find an alternate method to eradicate shrimp and/or work around the shrimp. A water jet sled was built as an alternate method to eradicate shrimp from the oyster beds. An array of water nozzles is mounted on a sled that is towed over the ground by an oyster boat. A high volume of water at up to 150 PSI is directed into the substrate to kill shrimp or expose them to predators. The design is still in progress, but preliminary results of testing are encouraging. We are testing an alternative growing method for the ability to disrupt newly settled shrimp. By using large seed on cultch or single oyster seed, the ground can be dredged more frequently. We will test for the effect of frequent dredging to inhibit shrimp recruitment. Growing single oysters is another option. Single oyster production increases the dollar yield per acre. Less acreage is required, and more money per acre can be spent on shrimp reduction. We are testing kumamoto, *Crassostrea sikamea*, and eastern oysters, *C. virginica*, in addition to Pacific oysters, *C. gigas*, for their higher dollar value. We are considering off-bottom culture for these valuable species.

GROWTH OF CULTURED GEODUCKS, *PANOPE ABRUPTA*, AT THREE INTERTIDAL SITES IN PUGET SOUND, WASHINGTON. Kurt Johnson, Taylor Shellfish Company, Shelton, WA 98584; Dan Cheney and Andrew Suhrbier, Pacific Shellfish Institute, Olympia, WA 98501.

Geoduck year classes from one to six years old were sampled over one to three years at three intertidal geoduck farms in Puget Sound Washington. Clams were measured for shell length and wet weight. At age three years and greater, one site showed significantly greater growth than the other two. Differences among the sites are discussed including current, food supply, density of clams, and substrate type. The effect of geoduck density on growth was investigated in three- and four-year-old geoducks. Density was measured on a scale of several square meters. At the two sites with lower growth, a regression of size to density showed smaller clams at the higher densities. Evaluation of the density effect will allow growers to adjust their planting densities for optimum growth. While substrate type was not quantified in this study, our observations indicate that substrate is very significant in determining geoduck growth rates.

Geoducks can be considered to be planted too densely in areas where geoduck density slowed growth. Using the biomass per square meter for clams close to harvest size in dense areas, we can estimate the carrying capacity for farm production purposes. This

number can be converted to a planting density to obtain the target harvest size clam within the desired time period.

DETECTION AND PRELIMINARY EVALUATION OF PARASITIC NATURAL ENEMIES FOR CONTROL OF BURROWING THALASSINID SHRIMP. Armand Kuris, M. Torchin, E. Ashley, S. Heerhartz, Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA 93106; and B. Dumbauld, Washington State Department of Fish and Wildlife, Willapa Bay Field Station, Ocean Park, WA 98640.

An assessment of the presence and prevalence of native natural parasitic enemies of two species of burrowing thalassinid shrimp was conducted to determine whether there was any potential for augmentative biologic control. The shrimp cause significant problems for oyster aquaculture in West Coast estuaries by undermining the substrate that oysters grow on. Results were compared with a similar survey on ghost shrimp from southern California and northern Baja California. Ghost shrimp from Willapa Bay and Grays Harbor, Washington, were infected with two species of larval nematodes that are trophically transmitted, probably via teleost fish, whereas ghost shrimp from California were parasitized by trematode metacercariae and larval tapeworms (not found in Washington). Mud shrimp from Washington estuaries were heavily infected with a bopyrid isopod in the gill chamber. Prevalence was extremely high (>85%) at some locations and data collected at one site during the last 8 y suggests that prevalence has increased markedly over time while the population of shrimp has declined over the same period. Though no causal relationship can be established from this data alone, there is clearly some potential and further research is warranted.

NEXT STEPS FOR THE MOLLUSCAN BROODSTOCK PROGRAM: IMPROVING PACIFIC OYSTER BROODSTOCK FOR THE WEST COAST INDUSTRY. Chris Langdon, Sean Matson, John Brake, Drew Mosher, Ford Evans and David Stick, Coastal Oregon Marine Experiment Station and Department of Fisheries and Wildlife, Oregon State University, Newport, OR 97365.

The Molluscan Broodstock Program (MBP) was established in 1995 to improve yields of Pacific oysters on the West Coast of the United States through family-based selection. Yields of F_1 families derived from selected P_1 families were 9.5% greater than those of nonselected control families. "Generalist" families were identified that consistently performed well across a range of different grow-out sites. Shell shape as well as shell and mantle pigmentation are, in part, genetically determined, and these traits could be enhanced to improve product value in "boutique" markets. Commercial hatcheries are now using MBP broodstock for large-scale production. There is a need to develop a commercial repository to ensure

adequate future supplies of broodstock for the West Coast industry.

BETWEEN A ROCK AND A HARD PLACE: THE ECOLOGY OF OVIGEROUS GREEN CRAB, *CARCINUS MAENAS* (L.), WITH EMPHASIS ON IMPLICATIONS FOR MONITORING AND CONTROL EFFORTS. P. Sean McDonald, G. C. Jensen, and D. A. Armstrong, School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195.

Efforts to eradicate nonindigenous species or control their spread may fail if management plans do not specifically focus on critical or susceptible life history stages of the target organism. The case of European green crab, *Carcinus maenas*, in the northeastern Pacific provides one such example. Control strategies often rely on baited traps that primarily capture male crabs because ovigerous (egg-bearing) females are less mobile and unresponsive to bait. Yet removing males is of little consequence in limiting populations; one male can mate with multiple females within a season, and sperm storage by the latter may allow them to fertilize many broods from a single encounter. The targeted removal of ovigerous *C. maenas* may be an effective method for reducing propagule pressure, but the lack of information regarding their habitat use and behavior has hindered efforts. We conducted intertidal surveys in Tomales Bay, California, in May/June 2001, and experiments were undertaken at Bodega Marine Laboratory, Bodega Bay, California, to investigate substrate preference and habitat competition with native brown rock crab, *Cancer antennarius*. Survey observations suggest that aggregations of ovigerous *C. maenas* occur under boulders or other structure at +0.11 to +0.41 m MLLW. These crabs also preferentially bury in medium/coarse sand under field and laboratory conditions. Results of a competition experiment and nighttime video observations indicate that *C. antennarius* may affect the distribution and habitat use of ovigerous *C. maenas* in some areas. Implications for monitoring and control efforts are discussed as well as recommendations for future work.

ENVIRONMENTAL CONDITIONS AND EXPERIMENTAL INFECTION WITH *PERKINSUS MARINUS* MODULATE CELLULAR DEFENSE MECHANISMS IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. Michael Goedken and Sylvain Deguise, Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT 06269.

The fast-growing oyster aquaculture industry is greatly hindered by the parasite *Perkinsus marinus*. The relationship between *P. marinus*, oyster defense mechanisms, and their environment is unclear. Apoptosis of Eastern oyster (*Crassostrea virginica*) hemocyte subpopulations was quantified at the single-cell level using flow cytometric Annexin-V and TUNEL assays. The influence of salinity (540 mOsm and 910 mOsm) and temperature

(14.50°C and >21.50°C) were evaluated experimentally on both the host and the parasite. The *in vivo* and *in vitro* effects of *P. marinus* infection on oyster hemocytes were measured as well. Forward and side scatter distinguished two populations of hemocytes (granulocytes, hyalinocytes) with previously demonstrated unique functional characteristics. Controlled environment and infection studies revealed significant defense mechanism alterations. Apoptosis was always higher in granulocytes than hyalinocytes regardless of temperature or salinity. Salinity affected apoptosis of both subpopulations with increased apoptosis at high as compared with low salinity irrespective of temperature. Parasite apoptosis was significantly higher at low salinity. *In vivo P. marinus* infection of oysters did not significantly affect hemocyte apoptosis, though *in vitro* infection resulted in greater apoptosis in infected granulocytes. We demonstrated that water salinity and experimental infection with *P. marinus* but not water temperature modulated oyster defense mechanisms. A better understanding of how these variables affect oysters defense mechanisms may lead to management strategies that will result in reduced disease morbidity and mortality for oyster producers.

ANALYSIS OF LARVAL AND JUVENILE PERFORMANCE IN CROSSBRED LINES OF PACIFIC OYSTERS, *CRASSOSTREA GIGAS*, UNDER THREE FEEDING REGIMES. Lizzie Nelson, Carolyn Friedman, School of Aquatic and Fishery Sciences, University of Washington, Seattle, WA 98195; Jonathan Davis, Taylor Resources, Inc., Quilcene, WA 98376; and Dennis Hedgecock, Department of Biologic Sciences, University of Southern California, Los Angeles, CA 90089.

Pacific oysters exhibit heterosis, the positive effect of heterozygosity seen in hybrid families. The goals of this study were to determine if rank of hybrid and inbred families varies as a function of food level, to examine within-family variation in growth and survival, and to investigate the relationship between larval and juvenile growth. Three inbred and six hybrid genotypes were replicated either in three food levels or by using three parent pairs per cross. Live wet weights and counts were taken throughout the experiment. Mortality, shell area of larvae (μm^2), and specific growth rate (SGR) will be analyzed. Rank of hybrid and inbred families' SGRs did not vary as a function of food level ($P = 0.006$) from days 39–67 postfertilization. Results after growout in Thorndyke Bay, Washington, are presented with an examination of the role of larval performance in adult yield prediction.

TRIALS OF ALTERNATIVE CHEMICAL AND MECHANICAL TACTICS TO MANAGE BURROWING SHRIMP—

2003 Kim Patten, Wsu Long Beach Res & Ext. Unit, Long Beach, WA 98631; **James Durfey**, Department of Biomechanical Engineering, Pullman, WA 99164; and **Steven Booth**, Willapa Bay-Grays Harbor Oyster Growers Association, Nahcotta, WA 98637.

Summer 2003 was the second experimental season to feature a series of randomized block designs using small field plot (2 × 2 m) to compare EPA 25-B list ("easily registered") compounds, bio-rational compounds, adjuvants, and reduced rates of the conventional burrowing shrimp compound, carbaryl (Sevin @ 8 lb a.i./ac, Bayer Corp.). Experimental materials were applied using a CO₂ backpack sprayer. A separate larger strip-plot (2 × 6 m) trial featured additional treatments of subsurface injection and postapplication rolling or harrowing. In all trials, shrimp burrows per m² were counted periodically after treatment and were compared using ANOVA. None of the unconventional treatments reduced burrow densities below the generally accepted threshold for oyster production of 10 per m. In an initial trial, burrow density was significantly lower in plots treated topically with acetamiprid (Assail @ 4.7 oz a.i./ac) compared with either carbaryl (2 lb a.i./ac) or the untreated check. These results contrast with those of the larger strip plot trial, however, where shrimp burrow densities were lower in carbaryl-treated plots (3 lb a.i./ac) than in acetamiprid-treated plots (14.4 oz a.i./ac). Burrow densities were initially lower in plots treated topically compared with injection, but differences were not significant at 4 wk after application. Effects from post-application rolling did not differ significantly from those due to postapplication harrowing.

METHODOLOGY OF DUNGENESS CRAB ASSESSMENT IN BRITISH COLUMBIA. Antan Phillips

, Fisheries and Oceans Canada, Pacific Biologic Station, Nanaimo, BC, Canada V9T 6N7.

Dungeness crab (*Cancer magister*) stocks on the Pacific coast have been sustained for well over one hundred years through minimum harvest size regulation alone. Assessment activities are therefore not directed at establishing harvest quotas but at improving fishery viability and providing access to the resource for all stakeholders. Assessments vary with information requirements and incorporate a variety of information sources including harvest logs, sales slips, and commercial sampling in addition to directed survey data. Assessment methodologies are described as they are applied to specific investigations and data requirements. Biological assessment focuses on analysis of trap-caught crabs either from commercial fisheries or from directed surveys conducted by Fisheries & Oceans Canada using standardized gear. Detailed information regarding harvest parameters as well as biological information on individual crabs is collected according to a standard format. A protocol for the collection of biologic information on crabs and the logistics of field data collection are presented.

WASHINGTON DEPARTMENT OF FISH AND WILDLIFE SHELLFISH DISEASE, PEST, AND PREDATOR CONTROL PROGRAM. Russell E. Rogers, Jr.

Washington State Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, Brinnon, WA 98320.

The Washington Department of Fish and Wildlife (WDFW) controls the introduction of new shellfish diseases, pests, and predators through the issuance of Shellfish Import Permits. The spread of diseases, pests, and predators already established in the state are controlled by the issuance of Oyster and Shellfish Transfer Permits. Recent changes in the status of established diseases, pests, and predators (Denman Island Disease [DID], Japanese oyster drill, and European green crab) has warranted some new restrictions on the intrastate transfer of shellfish. Following the discovery of DID, in Dungeness Bay, Clallam County, Washington, in May 2002, WDFW quickly established a surveillance program to monitor the disease. Samples were gathered from 18 sites in 2002 and 2003. At least seven additional sites (Westcott Bay, Deer Harbor, East Sound, and West Sound [all in San Juan County], Bellingham Bay, Samish Bay, and McMicken Island State Park) have been identified as positive for the disease. Data collected from the DID surveillance program has been used to establish DID prohibited and DID surveillance. Transfers within the surveillance areas will now require a written permit from WDFW. Surveys for Japanese oyster drills have identified five new infested areas (Duckabush river mouth, Jorsted Creek, the hook of the Hood Canal, Vaughn Bay, and Minterbrook Creek). These areas are now considered drill-restricted areas. Due to budgetary constraints, the European Green Crab Control Program was cut at the end of June 2003. The Puget Sound volunteer monitoring effort may continue if alternative funding can be secured.

ASSESSMENT AND MANAGEMENT OF SPOT PRAWN, *PANDALUS PLATYCEROS*, ALONG THE BRITISH COLUMBIA COAST. Dennis T. Rutherford, H. Nguyen, J. Boutillier

, Fisheries and Oceans Canada, Pacific Biologic Station, Nanaimo, BC, Canada V9T 6N7; and **J. Morrison**, Fisheries and Oceans Canada, Nanaimo, BC, Canada V9T 1K3.

Prawns, *Pandalus platyceros*, are the largest of the seven species of shrimp harvested along the coast of British Columbia. The commercial trap fishery accounts for more than 95% of the total prawn landings and represents the most valuable shrimp fishery in British Columbia. Landed values during the past few years have been in excess of \$25M Cdn. The prawn by trap fishery is managed by Fisheries and Oceans Canada to prevent both growth and recruitment overfishing. Growth overfishing is controlled through a combination of seasonal closures, size limits, trap mesh size requirements, fishing time restrictions, and a single haul limit. Recruitment overfishing is controlled using a fixed escapement-based model. Escapement is indexed based on the number of female spawners per trap. Implementation of the fixed escapement

strategy is carried out through an in-season, industry-funded monitoring program. The assessment and management frameworks for prawns appear to have been fairly successful; nevertheless, the fixed escapement model still requires improvements to achieve optimum production from all areas of the coast.

USING GIS TO ENHANCE UNDERSTANDING OF SPATIAL AND TEMPORAL INTERTIDAL CLAM DISTRIBUTION PATTERNS IN NORTH PUGET SOUND. Catherine A. Stanley and Bridget C. Gregg, Washington Department of Fish and Wildlife, Region 4 Shellfish, Mill Creek, WA 98012.

Geographic Information Systems (GIS) mapping technologies can be used to enhance spatial and temporal understanding of biologic systems. During annual clam population surveys, Washington Department of Fish and Wildlife (WDFW) shellfish biologists set transect lines at 100-foot intervals across the clam-band of a beach and collect 1-ft.³ samples at regular 40-ft. intervals along each transect line. Data recorded includes the GPS waypoints, species, number, length, and weight of clams and substrate type for each sample. This year, a pilot project was implemented to import these data into a GIS database using ArcView 3.2. Resource maps were created for several of the most popular beaches in North Puget Sound, mapping the distribution of *Protothaca staminea*, *Venerupis philippinarum*, and *Saxidomus giganteus*. GIS facilitated post-survey stratification of clam species by tidal elevation and substrate strata. Subsequent statistical analysis that incorporated these strata demonstrated local habitat preferences of recreationally targeted clams.

CONCERN OF DECREASED RESPONSE WHEN ADDING CANDIDATE GENE INFORMATION TO SELECTION INDEX CONTAINING PHENOTYPIC DATA. David A. Stick, Department of Fisheries and Wildlife, Oregon State University, Hatfield Marine Science Center, Newport, OR 97365.

Technological advancements have made possible direct genotypic selection for quantitative traits. It has been postulated that incorporation of genetic information in a phenotypic-based selection scheme will increase genetic response (ΔG) as result of increased selection accuracy and decreased generation interval. A Monte-Carlo gene-level simulation program was created to mimic meiotic events and test this hypothesis. Three levels of heritability (h^2) were investigated: very low (0.01), low (0.1), and moderate (0.4), together with three selection schemes: phenotypic (P_C), genotypic (G_C), and a combination (C_{PG}) index of both information sources. Response to selection only benefited from incorporation of genotypic information when heritability was very low ($C_{PG} > P_C > G_C$). Selection based solely on phenotype demonstrated the greatest response when heritability was low or moderate ($P_C > C_{PG}$

$> G_C$). Across all heritabilities, favorable polygenic frequency increased more with phenotypic selection ($P_C > C_{PG} > G_C$), whereas both the number of favorable alleles lost due to random genetic drift and the rate of inbreeding were greatest with genotypic selection ($G_C > C_{PG} > P_C$). Index optimization significantly reduced the difference in predicted response between phenotypic and combined selection approaches, but general trends remained. Results demonstrate that placing too much emphasis on selection of candidate genes can be detrimental to breeding programs. However, incorporation of genetic information in optimized selection programs may help minimize loss of favorable alleles.

CAN OYSTERS AND EELGRASS CO-EXIST? INTERACTIONS BETWEEN *CRASSOSTREA GIGAS* AND *ZOSTERA MARINA* OUTSIDE AN AQUACULTURE SETTING. Heather M. Tallis, J. L. Ruesink, Biology Department, University of Washington, Seattle, WA 98195-1800; Lorena M. Wisehart, S. D. Hacker, School of Biologic Sciences, Washington State University Vancouver, Vancouver, WA 98686; and Brett R. Dumbauld, Washington State Department of Fish and Wildlife, Willapa Bay Field Station, Ocean Park, WA 98640.

Recent surveys of eelgrass (*Zostera marina*) in oyster (*Crassostrea gigas*) aquaculture areas have shown that aquaculture can decrease eelgrass density in Willapa Bay, Washington. Identifying which of the many aspects of oyster aquaculture is responsible for low eelgrass density is crucial to the advancement of sustainable culture practices. We conducted two types of oyster density manipulations in Willapa Bay during the past two years to assess the effects of oysters on eelgrass separate from other aspects of aquaculture, like disturbance during planting and harvest. Early results showed no significant relationship between oyster density and eelgrass growth. However, because some current aquaculture practices leave oysters on beds for multiple years, it is important to understand the long-term effects of oysters on eelgrass. We will discuss seasonal variation in eelgrass growth related to adult oyster density and annual variation in eelgrass growth related to spat density. Pore water nutrient levels were also measured to distinguish physical effects of oyster shell from biological effects of oyster waste production. These results will be related to eelgrass growth.

ASSESSMENT AND MANAGEMENT OF INTERTIDAL CLAM RESOURCES IN NORTHERN PUGET SOUND. Jennifer Whitney and Darcy Wildermuth, Washington Department of Fish and Wildlife, Region 4, Mill Creek Office, Mill Creek, WA 98012.

Puget Sound has a mix of publicly and privately owned tidelands, a complex situation for resource management. Since the

court order of United States District Court Judge Rafeedie in 1994 honoring treaties signed by Governor Stevens and Puget Sound tribes in 1854–1855, tribal governments and the Washington Department of Fish and Wildlife (WDFW) have co-managed intertidal shellfish on the more than 1300 public beaches in Puget Sound. Tribal harvesters, both commercial and subsistence, and non-tribal recreational harvesters split the allowable harvest 50:50. Public beaches are individually managed to provide both harvest opportunity and population stability for Manila and Native Little-neck clams, the primary species targeted by recreational harvesters. The Tribes and the WDFW follow an established random transect survey protocol to estimate clam populations. The total allowable catch (TAC) is set at 33% of Manilas >38 mm and 25% of Natives >38 mm. WDFW sets seasons and bag limits to keep recreational harvest at or below the recreational share of the TAC. Recreational effort is monitored by aerial surveys that count harvesters within a one-hour window of low tide on a series of public beaches. Counts for individual beaches are expanded by a factor derived from on-the-ground ingress observations over the six-hour low-tide period. Recreational catch is estimated by conducting creel surveys as harvesters egress the beach. Effort and catch estimates are combined to yield an estimate of total annual recreational harvest for each beach. If 50% of the TAC for a beach is exceeded by recreational harvest, the overage is accounted for by shortening the season or closure the following year. Though recreational harvest estimation methods have been in place for more than 15 y, technological advances such as GIS are increasing the precision of our estimates.

THE EFFECTS OF DIFFERENT AQUACULTURE TECHNIQUES ON *ZOSTERA MARINA* BIOMASS, DENSITY, AND GROWTH RATES IN WILLAPA BAY, WASHINGTON. Lorena M. Wischart, S. D. Hacker, School of Biologic Sciences, Washington State University Vancouver, Vancouver, WA 98686; Heather M. Tallis, J. L. Ruesink, F. Oyarzun, Biology Department, University Of Washington, Seattle, WA 98195-1800; and Brett R. Dumbauld, Washington State Department of Fish and Wildlife, Willapa Bay Field Station, Ocean Park, WA 98640.

Influence of shellfish aquaculture on eelgrass has been understudied in Pacific Northwest estuaries. In an effort to quantify possible positive and negative effects of shellfish aquaculture, we investigated the relationship between oyster culture type and eelgrass at three sites in Willapa Bay, Washington. At each site, we sampled an off-bottom long-line culture area, a dredged ground culture area, a handpicked ground culture area, and an area without aquaculture. We measured the standing biomass, percent cover and growth rate of eelgrass, as well as the density of vegetative and flowering shoots. In general, we found the largest growth rates in areas with off-bottom culture and those without aquaculture; these areas also had the greatest eelgrass biomass, density, and percent cover. Eelgrass growth and biomass were lower in handpicked and dredged culture areas and did not significantly differ from one another. Interestingly, there were significant site and culture type interactions for most variables suggesting that site-specific conditions may be as influential as culture technique in determining eelgrass growth.

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JOURNAL OF SHELLFISH RESEARCH

VOLUME 23, NUMBER 3

DECEMBER 2004



The Journal of Shellfish Research
(formerly *Proceedings of the National Shellfisheries Association*)
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Journal of Shellfish Research

Volume 23, Number 3

ISSN: 0730-8000

December 2004

www.shellfish.org/pubs/jsr.htm

POPULATION BIOLOGY OF THE PACIFIC GEODUCK CLAM, *PANOPEA ABRUPTA*, IN EXPERIMENTAL PLOTS, SOUTHERN BRITISH COLUMBIA, CANADA

A. CAMPBELL,¹ C. W. YEUNG,¹ G. DOVEY² AND Z. ZHANG¹

¹Pacific Biological Station, Nanaimo, BC, Canada V9T 6N7; ²West 123° Resource Consulting Inc., PO Box 781, Ladysmith, BC, Canada V9G 1A6

ABSTRACT Information was presented on the density, recruitment to harvestable size, and growth of the Pacific geoduck clam, *P. abrupta*, in 0.25 ha experimental plots with controlled fishing in two areas of southern British Columbia over a 9-year period. In two plots that had high exploitation in the first 3 years, the geoduck population densities and the average age (by removing old individuals) were substantially reduced, but thereafter densities slowly increased due to geoduck recruitment. Recruitment was similar between plots after high and low exploitation rates, suggesting that fishing and different geoduck densities had no long term effect on subsequent recruitment. After removing many old individuals from a geoduck population, the mean maximum asymptotic length (L_{∞}) of the von Bertalanffy growth model decreased, and the Brody growth function k values increased. In plots where there was low exploitation and geoduck densities remained similar over time, L_{∞} values also remained similar, but k values varied with time suggesting other biologic or environmental factors could have influenced growth rates.

KEY WORDS: geoduck, *Panopea abrupta*, density, recruitment, growth

INTRODUCTION

The Pacific geoduck clam, *Panopea abrupta* (Conrad, 1849), is a large hiatellid bivalve found buried up to 1 m within mud and sand substrates. This geoduck species is distributed in coastal areas from the intertidal zone to at least 100 m (Jamison et al. 1984) from California to Alaska and to southern Japan (Coan et al. 2000). Commercial fisheries occur for *P. abrupta* in Washington State, British Columbia and Alaska (Campbell et al. 1998a, Bradbury & Tagart 2000, Hand & Bureau 2000) with annual landed values varying between 32 and 42 million Canadian dollars in British Columbia during 2000 to 2003. Adult geoduck generally have separate sexes and broadcast spawn usually during summer (Andersen 1971, Sloan & Robinson 1984, Campbell & Ming 2003). Planktonic larvae remain in the water column 16 to 47 days, settle as postlarvae on substrates, and subsequently burrow into suitable substrates (King 1986, Goodwin & Pease 1989). Geoduck juveniles and adults feed by filtering food particles (e.g., phytoplankton) from seawater (Goodwin & Pease 1989). Geoduck growth is variable, depending on a variety of environmental factors, but fastest during their first 10 y (Goodwin & Shaul 1984, Goodwin & Pease 1991, Harbo et al. 1983, Bureau et al. 2002, Noakes & Campbell 1992, Hoffmann et al. 2000). Geoduck are long-lived, reaching ages of up to 168 y (Bureau et al. 2002). Geoduck recruitment was considered to be variable but low (Breen & Shields 1983, Harbo et al. 1983), however recent studies of age frequencies have suggested strong recruitment events to adult geoduck populations throughout BC since 1988 (Bureau et al. 2002, Bureau et al. 2003). Natural mortality rate (M) estimates of adult geoduck in British Columbia range from 0.01–0.04 (Breen & Shields 1983, Harbo et al. 1983, Noakes 1992).

Little is known of the effects of fishing on the population biology of *P. abrupta* (e.g., Goodwin 1978, Campbell et al. 1998a). The objective of the study was to investigate the effects of fishing on geoduck population biology. The purpose of this study is to present information on the density, recruitment to harvestable size, and growth of *P. abrupta* in small experimental plots with controlled fishing in two areas of southern British Columbia over a 9-year period. Further analyses of geoduck recruitment and natural mortality of this study are presented in Zhang and Campbell (2004).

Experimental Plots

The study areas were located at Ritchie Bay (northwest Meares Island on the west coast of Vancouver Island) and southwest Marina Island (Georgia Strait) of British Columbia. Ritchie Bay has been closed to commercial fishing and designated as a research reserve since 1980, when Fyfe (1984) started his geoduck study. The area around Marina Island was heavily harvested during 1978–1989 and was subsequently closed to commercial fishing for geoduck since 1990 (Campbell et al. 1996b). For this study, 4 experimental plots were established at Ritchie Bay [(1) 125°55.242'W, 49°13.502'N; (2) 125°55.179'W, 49°13.506'N; (3) 125°55.076'W, 49°13.488'N; (4) 125°55.046'W, 49°13.549'N] during 1991 and 3 plots established at Marina Island [(1) 125°03.734'W, 50°03.001'N; (2) 125°03.745'W, 50°02.959'N; (3) 125°03.826'W, 50°02.955'N] during 1992.

Each experimental plot comprising a 0.25 ha area (50 × 50 m), was delineated and subdivided into 10-m sections with lead lines to provide five 500 m² strips (50 × 10 m). Experimental plots 1, 2, and 3 at Ritchie Bay were slightly smaller (0.244 ha) than the other plots because 0.006 ha/plot were reserved for potential show factor plots (see later). The minimum-maximum depth (m) at datum for each experimental plot at Ritchie Bay was (1) 7.8–11.8; (2) 10.0–10.9; (3) 7.7–9.8; and (4) 10.7–11.6 and at Marina Island (1) 11.7–14.5; (2) 11.6–15.4; and (3) 12.2–16.8. The bottom substrates comprised of soft mud to fine sand and shell mixture at Ritchie Bay and fine sand with some small gravel at Marina Island.

Experimental Treatments

The chronology of events for each plot and year included an abundance (show factor and density) survey, biosample, and or commercial harvest (Table 1). Each plot was randomly chosen for a different treatment in terms of a depletion harvest regimen (Table 1, 2). The general harvest regimen in each plot for Ritchie Bay was (1) moderate, (2) none, (3) heavy, and (4) none, and for Marina Island was (1) none, (2) moderate, and (3) heavy. In an attempt to determine if seeding of hatchery-raised juvenile geoduck could supplement natural recruitment. Plot 1 and Plot 2 of Ritchie Bay,

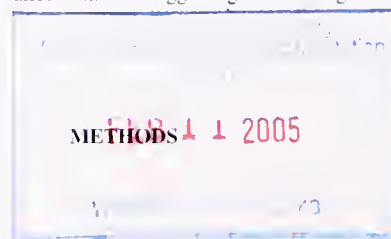


TABLE 1.

Annual schedule of abundance survey (A), bio-sampling (B) and or commercial harvest removal (C), and hatchery-reared geoduck juvenile seeding (E) in each experimental plot of the Ritchie Bay and Marina Island study area.

Year	Ritchie Bay Experimental Plot Number and Harvest Regime				Marina Island Experimental Plot Number and Harvest Regime		
	1 Moderate	2 None	3 Heavy	4 None	1 None	2 Moderate	3 Heavy
1991	A, B, C, E	A, E	A, C	A			
1992	A	A	A, B, C	A	A	A, C	A, B, C
1993	A	A	A, C	A	A	A	A
1994					A	A	A
1995	A, B	A, B	A	A, B			
1996	A	A	A	A	A	A	A
1997	A	A	A	A	A	A	A
1998							
1999					A	A	A
2000	A, B	A, B	A, B	A, B			
2001					A, B	A, B	A, B

The chronological sequence of events for each plot and year is shown in alphabetical order.

were artificially seeded at approximately 16 m^{-2} with younger then 0.5-y-old hatchery-raised juvenile geoduck of mean 11.3 mm shell length (SL) (min. 6 mm SL, max. 18.4 mm SL, $n = 136$) during September 1991. Plot 4 of Ritchie Bay and Plot 1 of Marina Island were considered the control treatment as the only removals made were for biosamples and were not seeded with hatchery-raised juvenile geoduck. No treatment was replicated with addi-

tional plots in the same study area because of the considerable logistical costs involved.

Densities

Geoduck density was estimated using survey methods described by Campbell et al. (1996 a, 1996 b). The survey method for

TABLE 2.

Summary of pre-harvest geoduck densities, number and density of geoduck removed, through harvest and or biosample removals, and exploitation rate, by experimental plot at the Ritchie Bay and Marina Island study areas.

					Geoduck Density (no.m ⁻²)			Exploitation Rate		
		Removed total no.	Biosample total no.	Removed density no.m ⁻²	Mean	95% CI		Mean	95% CI	
Plot	Year					Lower	Upper		Lower	Upper
Ritchie Bay										
1	1991	5580	624	2.29	3.67*	2.84	5.06	0.62	0.45	0.81
	1995	532	532	0.22	3.17	2.82	3.58	0.07	0.06	0.08
	2000	508	508	0.21	2.00	1.75	2.27	0.10	0.09	0.12
2	1995	506	506	0.21	7.35	6.87	7.86	0.03	0.03	0.03
	2000	494	494	0.20	5.72	5.35	6.09	0.04	0.03	0.04
3	1991	2930	—	1.20	2.54	2.33	2.75	0.47	0.44	0.51
	1992	4024	489	1.65	1.55	1.34	1.77	1.06	0.93	1.23
	1993	183	—	0.08	0.15	0.10	0.19	0.51	0.39	0.76
	2000	448	448	0.18	0.76	0.65	0.88	0.24	0.21	0.28
4	1995	521	521	0.21	3.85	3.54	4.15	0.05	0.05	0.06
	2000	516	516	0.21	2.68	2.45	2.91	0.08	0.07	0.08
Marina Island										
1	2001	417	417	0.17	0.17	0.13	0.22	0.96	0.75	1.33
2	1992	878	—	0.35	0.34	0.27	0.41	1.04	0.86	1.12
3	2001	291	291	0.12	0.14	0.10	0.19	0.82	0.63	1.11
	1992	1356	556	0.54	0.71	0.61	0.83	0.76	0.66	0.89
	1993	334	—	0.13	0.33	0.23	0.53	0.40	0.25	0.59
	1994	163	—	0.07	0.12	0.07	0.17	0.56	0.39	0.88
	2001	330	330	0.13	0.18	0.14	0.22	0.73	0.60	0.92

Plots 1, 2, and 3 from Ritchie Bay were 0.244 ha/plot, the rest were 0.250 ha/plot. Removed total and density includes the harvest and biosample removals. no, number; CI, confidence interval.

each plot involved counting geoduck siphons in 5-m² (5 × 1 m) quadrats along ten 50-m one meter wide transects. Two transects were placed on either side of the four inner subdivision boundaries and one transect each was placed on the two inner sides of the study area border for a total of 10 transects per plot. Six of the eight inner transects for Plots 1, 2, and 3 in Ritchie Bay were shortened because they had 10 m reserved as potential show factor plots, where no geoduck was removed. Consequently, 10 m² for each of these 6 transects per Plot 1, 2, and 3 were not used in the density estimates for Ritchie Bay. The total area considered for geoduck density surveys and harvest was 2440 m² for Ritchie Bay Plots 1, 2, and 3 and 2500 m² for Ritchie Bay Plot 4 and all three plots of Marina Island. There were 88 quadrats surveyed for Ritchie Bay Plots 1, 2, and 3 and 100 quadrats surveyed for Ritchie Bay plot 4 and Marina Island Plots 1, 2, and 3 for each density survey.

Survey dates for geoduck densities at Ritchie Bay were July 25 to August 14 1991; May 13–27, 1992; May 17–19, 1993; May 17–19, 1995; May 31 to June 1, 1996; May 24–25, 1997; July 9–10, 2000; and at Marina Island were July 15, 1992; July 8–9, 1993; June 6, 1994; June 4–5, 1996; September 18–19, 1997; October 5, 1999; September 7–8, 2001 (Table 1).

Show Factor

Because all geoduck may not show their siphons at the substrate surface at any one time, "show" plots were used to determine the percentage of exposed geoduck (i.e., showing their siphons each day). In Ritchie Bay, only the three show plots in Plot 2 were used throughout this study. In the Marina Island study area, the three show plots were placed 5–10 m outside the borders of the three experimental plots. Each 20 m² show plot (i.e., 2 × 10 m, subdivided into four 1 × 5 m quadrats) was marked with lead line providing a total of 12 quadrats in the three show plots of each study area. Each geoduck showing in the show plots was marked beside the neck with a "flag" (stainless steel wire) in the substrate. The proportion of geoduck showing (the show factor) on any given day was determined by dividing the number showing on that day by the cumulative total number of geoduck flagged during the period that the show plots were monitored each year. Monitoring of the show plots started several days before the density surveys were conducted in the experimental plots. Sometimes not all the show plots were monitored. On a few occasions, less than three show plots were surveyed, and the available data were used for the analyses.

For most plots, both the shows and density were surveyed on the same day. However, for a few days some Ritchie Bay plots did not have show factor and density surveys on the same day, in which case the show factor used was interpolated (averaged) between dates. One exception to a reliable interpolation occurred for the geoduck density estimate at Ritchie Bay Plot 1 which was surveyed during August 7–14, 1991 when the show factor had been measured on July 26 and 31 and August 1 and 15, 1991 and estimated to be 0.96. This show factor value was clearly incorrect and too high because of a severe storm, with 115.6 mm of rain and south east winds of up to 43 km/hour, occurred on August 7, 1991 (Environment Canada web site for Tofino Airport [125°24'W, 49°4'N] http://www.climate.weatheroffice.ec.gc.ca/climateData/canada_e.html). The diver surveying for geoduck density on the August 15, 1991 also recorded that geoduck were not showing well due to a "winter like storm" the previous week. Campbell et al.

(1996a) found that show factor decreased to about 0.65 following a strong storm near Comox during June 1993. All measured show factors at Ritchie Bay were higher than 0.80. Show factor data from the Marina study area on July 8, 1993 also provided a reduced show factor of about 0.66, due to increased wind speeds (hourly weather and wind speeds and direction were examined for Campbell River Airport [125°16'W, 49°57'N; see website: http://www.climate.weatheroffice.ec.gc.ca/climateData/canada_e.html] a location near to Marina Island, during the survey periods and about 5 days prior to the survey). Consequently a show factor value of 0.66 was considered reasonable to be used in the density calculations for Ritchie Bay Plot 1 in 1991.

Removals

Geoduck were removed from each plot for two reasons: (1) to deplete the population through commercial harvest, and (2) to collect a biosample for aging. Removal of geoduck from each plot was always conducted soon after the density surveys had been completed. An experienced fisherman used the usual commercial harvest methods (Campbell et al. 1998a) to systematically harvest each of the five strips per plot right up to the rope borders on each day fished. This ensured, as much as possible (except for environmental influences such as tidal current and visibility variation), that all geoduck had an equal chance of being harvested in the plot on each day during the study. A target number for each biosample was a minimum of approximately 500 geoduck per plot (i.e., 100 per strip). Biosamples with 50 less than the 500 geoduck target were probably due to the divers not being able to find the extra individuals at low geoduck densities in the plots. When more than 1000 geoduck were removed per plot, the first approximately 500–600 geoduck were used as a biosample. Geoduck within the potential three 20 m² show factor plots in each of the Ritchie Bay Plots 1, 2, and 3, however, were not removed, making the actual area harvested 2440 m² per plot. The total number of geoduck removed per plot per year is shown in Table 2.

Biosamples

For each geoduck, the shell length (SL) was measured as the straight-line distance between the anterior and posterior margins of the shell to the nearest mm with veneer calipers. The age of each geoduck was estimated using the acetate peel method of Shaul and Goodwin (1982). One valve per geoduck was sectioned through the hinge plate, the cut surface polished, etched with a 1% hydrochloric acid solution for approximately 1 minute, washed with distilled water, dried, and an acetate peel made by applying first acetone, then an acetate sheet on the hinge surface. Annual growth rings imprinted on the acetate peel were counted on a digitizing table after ×40 magnification using a Neo-Promar projector. Shaul and Goodwin (1982) and Noakes and Campbell (1992) provide further details in shell preparation and age validation procedures. Although most individuals had both their SL and age measured, there were some that had only the SL or only the age measured; these latter individuals were included in the analysis only where appropriate.

To determine the weight of geoduck necks by age at recruitment, sub samples of between 45 and 115 geoduck, with as wide a size range as possible, were obtained from each biosample. For each geoduck, the neck was cut at the base from the body, cut longitudinally along the siphons to remove excess water and weighed in grams.

Data Analyses

Density

The mean show-adjusted density, D (number m^{-2}), for each plot was calculated as

$$D = \frac{\frac{1}{n} \sum_{i=1}^n d_i}{\frac{1}{m} \sum_{j=1}^m S_j} \quad (1)$$

where d_i is the density of geoduck (i.e., number of geoduck m^{-2} or number of geoduck per 1×5 m quadrat divided by 5) in the i^{th} quadrat, S_j is a proportion of geoduck showing in the j^{th} show quadrat, n is the total number of quadrats sampled to estimate geoduck density, and m is the total number of show quadrats (i.e., twelve 1×5 m quadrats) used for estimating show factor in each study area. Hereafter density in this study refers to show-adjusted density.

Distribution-free unbiased bootstrap techniques (Efron & Tibshirani 1993) with S-plus (2001) computer programs were used to estimate 95% confidence intervals (CI) for the mean show-adjusted density. Eq. 1 was used to obtain a new mean show-adjusted density by randomly resampling, with replacement, m new show factors from the original m show factors and n new density counts from the original n density counts. This procedure was repeated 1000 times. To produce the $(1-2\alpha)$ CI of show-adjusted density, the α percentile (2.5%) and the $1-\alpha$ percentile (97.5%) of the bias-corrected and accelerated (BCa) distribution of the 1000 show-adjusted densities were used as the lower and upper CI bound, respectively (Efron & Tibshirani 1993).

Exploitation

Exploitation was calculated as R/D , where R is the removed density (number of geoduck m^{-2} calculated as total number of geoduck divided by the plot area harvested), and D is the geoduck density (mean, 95% CI) surveyed just prior to removal.

Age Frequencies

The age frequency data were standardized for density to allow comparison of the number of geoduck m^{-2} belonging to a particular age group between plots and years. The equation used was:

$$D_a = p_a \times D \quad (2)$$

where D_a is the estimated mean density of geoduck of age a , p_a is the proportion of geoduck of age a (number of individuals of age a divided by total number of known age) in the bio-sample, and D is the mean show-adjusted density.

The 95% CI for density of age a were calculated using a bootstrap algorithm (Efron & Tibshirani 1993, S-plus 2001). Eq. 1 and 2 were used to obtain a new estimate by randomly resampling, with replacement, m new show factors from the original m show factors, n new density counts from the original n density counts, and k new age estimates from the original k age estimates. The m new show factors and the n new density count were used to estimate D , and the p_a was estimated by counting the number of age a in the k new age estimates. This procedure was repeated 1000 times. To produce the $(1-2\alpha)$ CI of show-adjusted density at age, the α percentile (2.5%) and the $1-\alpha$ percentile (97.5%) of the bias-corrected and accelerated (BCa) distribution of the 1000

show-adjusted densities were used as the lower and upper CI bound, respectively (Efron & Tibshirani 1993).

Pair-wise comparisons to test for differences in age frequencies between plots and years were conducted using the Mann-Whitney test.

The figures for age frequencies adjusted to density were truncated to 100 y for graphical purposes because there were few geoduck found older than 100 y in both study areas.

Recruitment

Recruitment was defined as the density of geoduck growing into the population that were fully vulnerable to fishing. We assumed recruitment to occur when all of the necks of geoduck of the youngest age cohort were large enough to be clearly seen by divers when showing at or above the substrate. The threshold criterion used for recruited geoduck neck size at the youngest age was where the lower 95% CI of the mean neck wet weight was equal to 100 g or higher. Recruitment for geoduck from the Ritchie Bay area was 6 y, (neck weight mean = 148.2, lower-upper 95% CI =

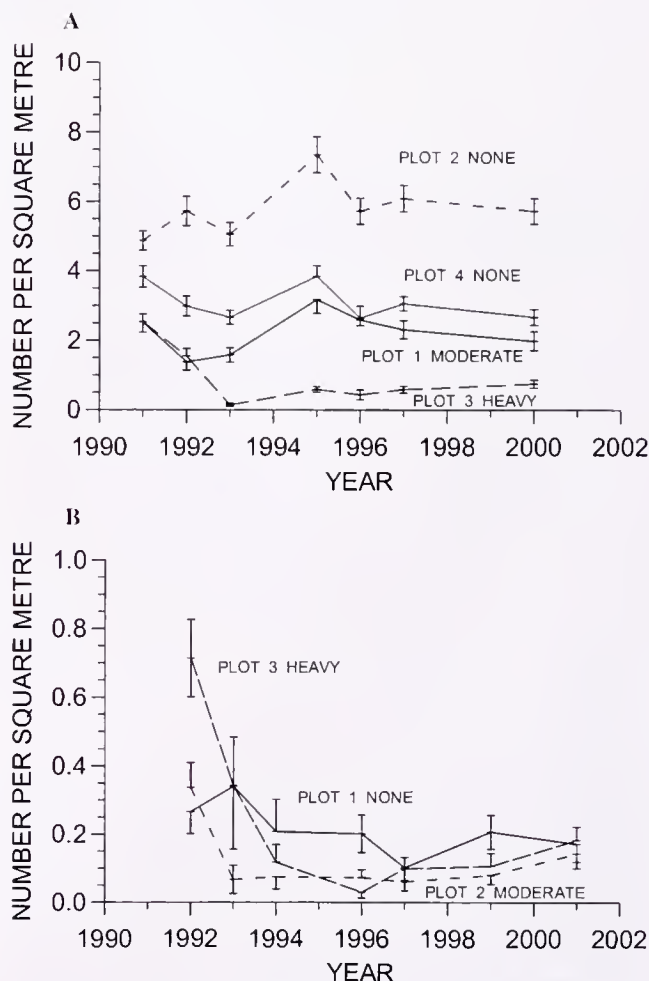


Figure 1. Density of geoduck in the study plots from (A) Ritchie Bay, 1991–2000, and (B) Marina Island, 1992–2001. Densities were adjusted for show factor. Vertical lines are upper and lower 95% CI; where there is an overlap of CI for two or more plots in a year only the upper or lower CI is shown for each plot. Harvest treatments were for Ritchie Bay plot (1) moderate, (2) none, (3) heavy, (4) none, and for Marina Bay plot (1) none, (2) moderate, (3) heavy.

130.5–165.9, $n = 34$), and from the Marina Island area was 7 y (neck weight mean = 124.2, lower-upper 95% CI = 104.2–144.2, $n = 22$). The densities of age 6 and 7-y geoduck and 95% CI for each plot were calculated as described in the previous section above. Because there were no replicates for each plot-treatment, for comparisons between plots and years, we considered overlapping 95% CI to suggest similarities in mean densities at age, and nonoverlapping 95% CI to suggest a difference in mean densities at age.

Growth

The relationship between shell length and age was estimated with the von Bertalanffy (1938) growth model

$$L_t = L_\infty[1 - e^{-k(t-t_0)}] + \varepsilon \quad (3)$$

where t is age in years, L_t is shell length (mm) at age t , L_∞ is the mean asymptotic length (mm), k is the Brody growth coefficient determining rate of increase or decrease in length increments, t_0 is

a phase-variable, suggesting the hypothetical age at which the organism would have been at zero length, and $\varepsilon \sim N(0, \sigma^2)$ is a normal variate. The parameters L_∞ , k , t_0 , and ε were estimated using maximum likelihood methods (Bain & Engelhardt 1991) in the statistics program of S-Plus (2001). Only undamaged geoduck shells were used for the growth analyses. Initial analyses indicated that t_0 values were either far too positively or negatively large in the biosamples that had few young geoduck. For consistency, we followed the same approach of Bureau et al. (2002) by setting t_0 as zero for all samples causing the fitted curves through the origin and re-estimating L_∞ , k , and ε .

The 95% CI for the parameters L_∞ , k , and ε from each biosample were calculated using a bootstrap algorithm as follows. Pairs of age length data for individual geoduck were randomly resampled, with replacement, from the original data up to the biosample size n , and then Eq. 3 (with $t_0 = 0$) was used to obtain estimates of the parameters. This procedure was repeated 1000 times. To produce the $(1-2\alpha)$ CI for each of L_∞ , k , and ε ; the α percentile (2.5%) and the $1-\alpha$ percentile (97.5%) of the bias-corrected and accelerated (BCa) distribution of the 1000 values of each parameter were used as the lower and upper CI bound, respectively (Efron & Tibshirani 1993).

Pair-wise comparisons for hypothesis testing of simultaneous differences in parameters L_∞ and k between all biosamples were conducted using the likelihood ratio test outlined by Kimura (1980) to determine if geoduck growth rates were different between plots and years.

Relation Between Density, Recruitment, and k

Regression analyses (using the least squares method) were used to determine if there were significant relationships between the Brody growth coefficient k and mean overall density or recruitment density, for each plot and year at Ritchie Bay.

TABLE 3.

Summary statistics of unmodified age data from the Ritchie Bay and Marina Island experimental plot biosample data.

		Age (years)					
Year	Plot	Mean	Median		(Min-Max)	SE	<i>n</i>
Ritchie Bay							
1991	1	34.1	34.0	a	(3–99)	0.87	522
1992	3	38.9	43.0	b	(3–111)	1.03	443
1995	1	24.0	16.0	c	(2–104)	0.94	512
1995	2	42.8	46.0	e	(1–123)	0.98	487
1995	4	34.4	35.0	a	(2–123)	1.07	495
2000	1	19.2	9.0	c	(3–90)	0.79	498
2000	2	37.1	41.0	b z	(3–115)	1.12	447
2000	3	12.9	7.0	f	(2–111)	0.76	443
2000	4	33.7	34.0	ay	(3–124)	1.14	507
Marina Island							
1992	3	36.7	30.0	a z	(1–125)	1.16	510
2001	1	30.2	19.0	y	(3–112)	1.19	411
2001	2	16.1	9.0	x	(3–98)	1.09	289
2001	3	13.8	8.0	w	(3–116)	0.88	325

SE = standard error of mean, n = number of geoduck sampled. For each study area, medians followed by the same letter indicated age distributions were not significantly different (Mann-Whitney pair wise test $p > 0.05$), medians followed by a different letter indicated age distributions were significantly different (Mann-Whitney test, $P < 0.05$).

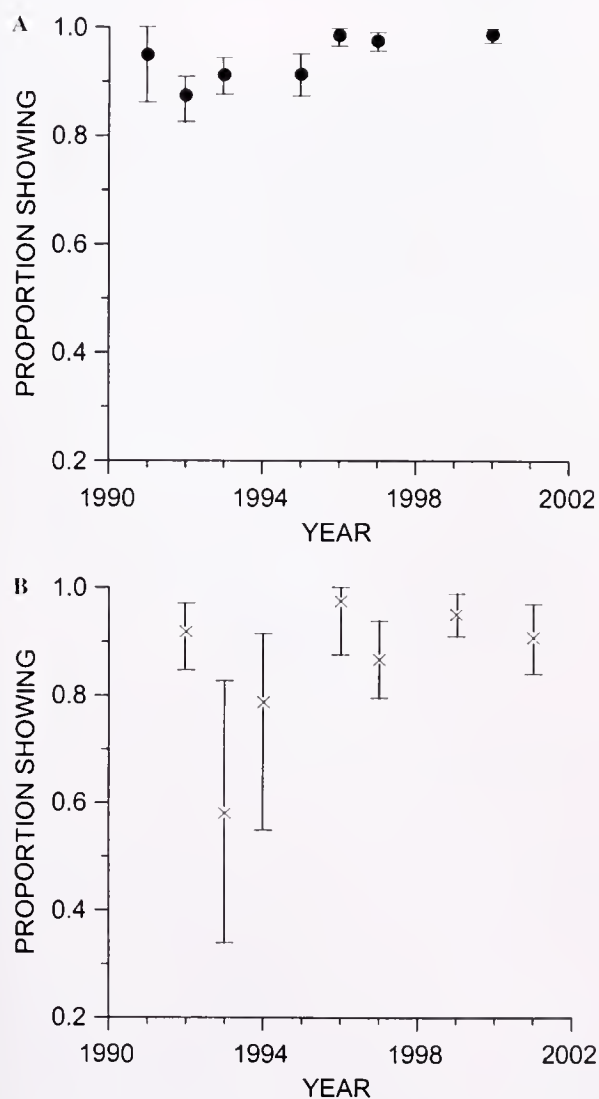


Figure 2. Proportion of geoduck necks showing in show factor plots (A) Ritchie Bay, and (B) Marina Island. Means (dots or x) and 95% CI (vertical lines) per year include 1–3 days of sampling.

RESULTS

Density

For Ritchie Bay, geoduck density remained highest over time in unharvested Plot 2 and lowest in the highly exploited Plot 3 (Fig. 1A). The removals over 3 y of practically all geoduck in Plot 3 caused geoduck densities to decline significantly by 1993 and to remain low, although there was a small density increase by 2000 (Fig. 1A). Although there was 0.62 exploitation rate of the geoduck in Plot 1 during 1991 (Table 2), with a consequent decline in geoduck density in 1992, densities recovered by 1995 (Fig. 1A). Geoduck densities in Plot 4 fluctuated between about 2.68 to 3.85 m^{-2} (Fig. 1A, Table 2).

Geoduck densities in the Marina Island experimental plots were considerably lower than those for Ritchie Bay (except for Plot 3 after 1992) (Fig. 1, Table 2). The removal of substantial numbers of geoduck reduced densities in both Marina Island Plots 2 and 3 (Fig. 1B, Table 2). Although Plot 3 had the highest and lowest density of the Marina Island plots in 1992 and 1996, respectively, Plot 3 densities recovered slightly to similar densities of control Plot 1 by 2001 (Fig. 1B, Table 2).

Exploitation rates were high especially in Plot 3 of Ritchie Bay and Marina Island, and in plots that had biosamples taken in already low density plots (Table 2). Mean estimates of exploitation values greater than 1.0 (i.e., Ritchie Bay Plot 3 in 1992, Marina Island Plot 2 in 1992) clearly were not possible and reflect the uncertainty of the density estimates of the survey method, but were possible within the lower 95% CI exploitation values (Table 2).

Show factors were generally recorded higher than 0.8 for most surveys in both study areas, except for Marina Island during 1993 to 1994 (Fig. 2) and for Ritchie Bay Plot 1 in August 1991 due to inclement weather (see also show factor in methods section). Strong winds, that could have affected geoduck to produce low show factors, were recorded at 30 and 28 km per hour from a northwest direction during July 5–6, 1993 respectively, and at 30 km per hour from a south east direction during June 4, 1994 near Marina Island. There were southeast winds of up to 43 km/hour recorded on August 7, 1991, near Ritchie Bay.

Age Frequencies

Following heavy harvesting there was a significant decrease in median ages (Table 3) and clear reduction in the densities of old geoduck in Ritchie Bay Plot 1 (Fig. 3A, 3B, 3C) between 1991 and 1995, Plot 3 (Fig. 3 F, 3G) between 1992 and 2000, and Marina Island Plot 3 (Fig. 4C, 4D) between 1992 and 2001. There was no significant change in geoduck median age for Ritchie Bay control Plot 4 between 1995 and 2000 (Table 3, Fig. 3H, 3I).

Recruitment

For Ritchie Bay, geoduck recruitment density at age 6 y, was generally higher for all plots in 2000 than in previous years (Table 4, Fig. 3). Recruitment within plots between years, was similar for Plot 1 between 1991 and 1995 but higher in 2000; was higher for Plot 2 in 2000 than 1995; higher for Plot 3 in 2000 than 1992; and similar for control Plot 4 in 1995 and 2000 (Table 4, Fig. 3). Geoduck recruitment density between Plots 1, 2, and 4 in 1995 was similar, and recruitment for all four plots in 2000 was similar.

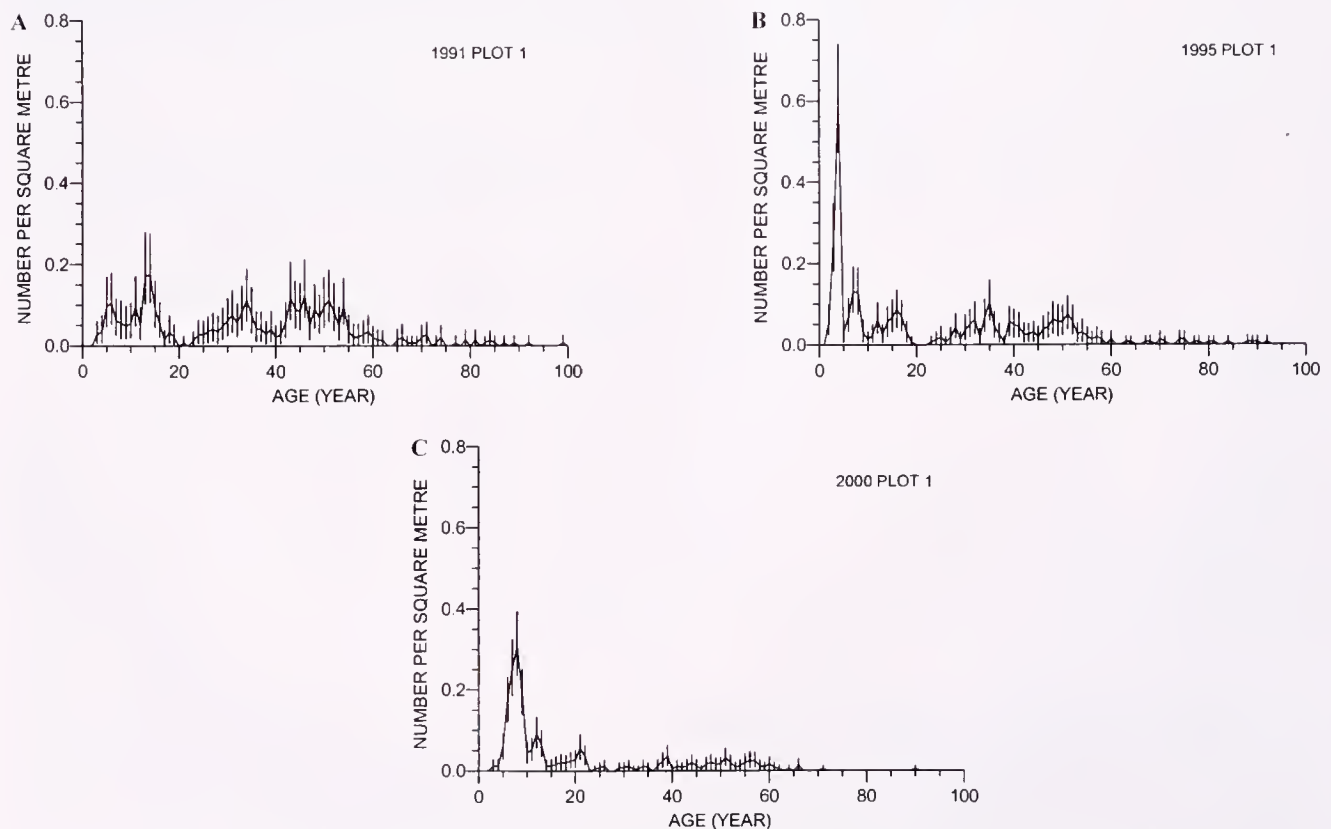


Figure 3. Density age frequencies for geoduck in the experimental plots of Ritchie Bay. Vertical lines are 95% CI.

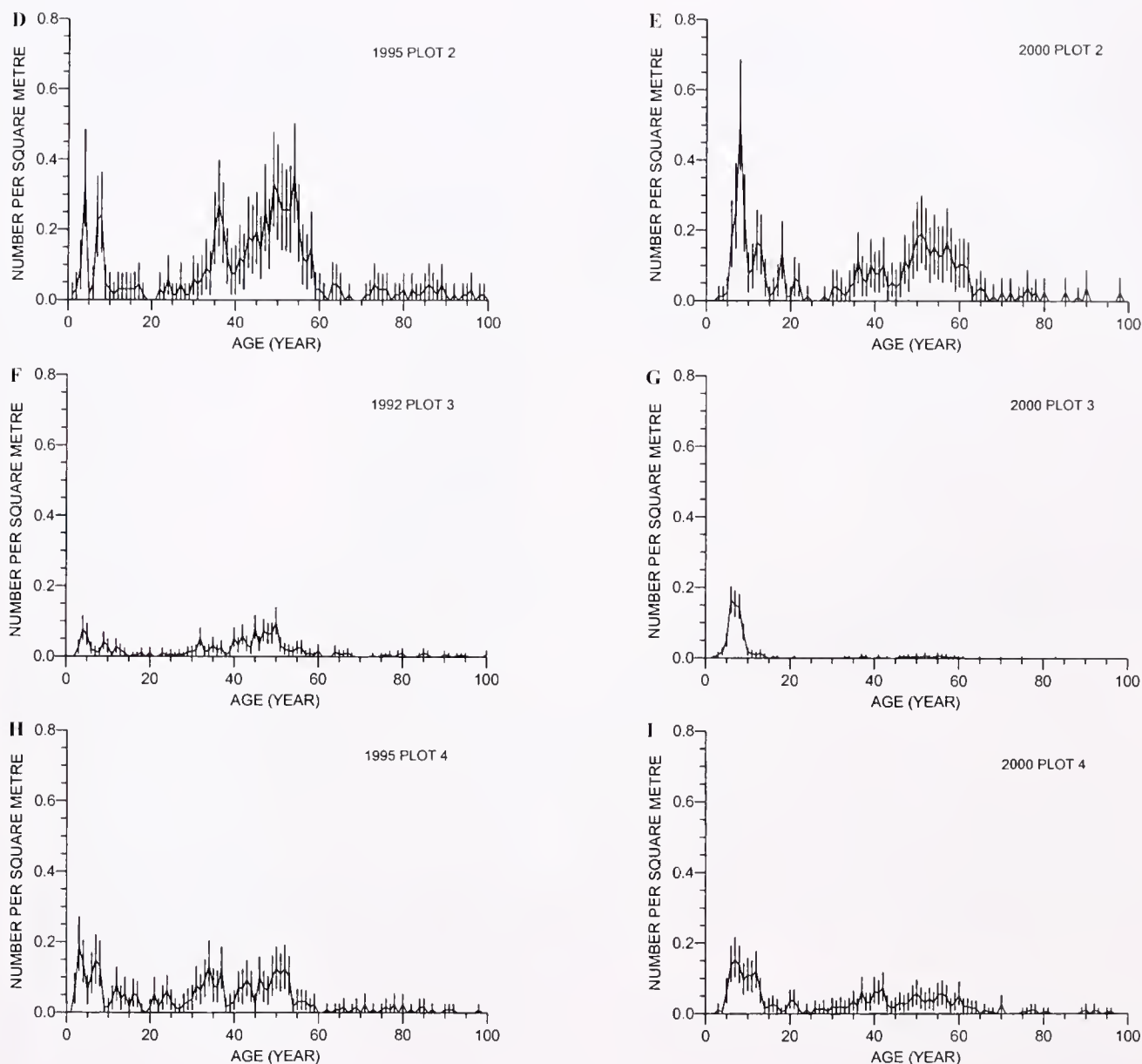


Figure 3. Continued.

For Marina Island, geoduck recruitment density at age 7 y, was slightly higher for Plot 3 in 2001 than 1992 (Table 4, Fig. 4). Plots 2 and 3 had higher geoduck recruitment than Plot 1 in 2001 (Table 4, Fig. 4).

Geoduck recruitment at Ritchie Bay was well above that for Marina Island in the last 10 y (Fig. 3, 4, Table 4). The highest density for each plot at a particular age and year: for Ritchie Bay Plot 1 was 0.60 at 4 y in 1995, 0.31 at 8 y in 2000; Plot 2 was 0.33 at 4 y in 1995, 0.51 at 8 y in 2000; Plot 3 was 0.08 at 4 y during 1992, 0.16 at 6 y in 2000; Plot 4 was 0.18 at 3 y in 1995, 0.15 at 7 y in 2000 (Fig. 3); and for Marina Island Plot 1 was 0.014 at 8 y in 2001; Plot 2 was 0.029 at 8 y in 2001; Plot 3 was 0.038 at 9 y in 1992, and 0.042 at 8 y in 2001 (Fig. 4).

The influence of artificial seeding on the density of the 1991 cohorts, 4 and 9 y later, was suggested by the higher geoduck densities in Plot 1 (4.28 and 1.90 times) and Plot 2 (2.36 and 2.40

times) compared with the geoduck densities in unseeded Plot 4 during 1995 and 2000, respectively (Table 5; Fig. 3).

Growth

The results on the geoduck growth curves (Table 6, Fig. 5) and the Kimura pair-wise comparisons (significant differences at $P < 0.05$) (Table 7) were grouped as follows.

Within Each Plot Between Years

The Brody growth coefficients, or k values, for geoduck in Ritchie Bay Plots 1, 2, and 4 were significantly higher in 1995 than for the same plots in 1991 and 2000. In contrast, the k value for Ritchie Bay Plot 3 was significantly higher in 2000 than in 1992. The k value for Marina Island Plot 3 was significantly higher in 2001 than 1992.

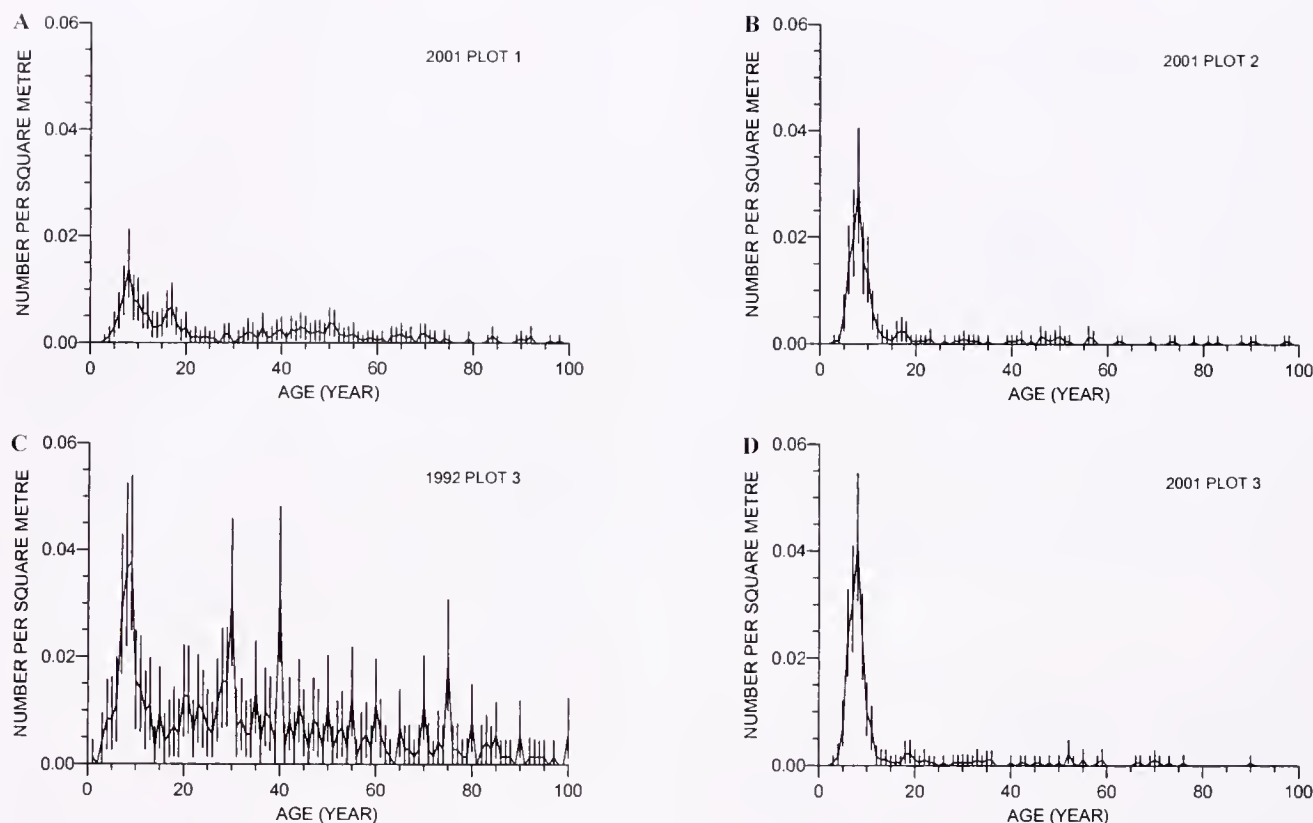


Figure 4. Density age frequencies for geoduck in the experimental plots of Marina Island. Vertical lines are 95% CI.

The L_{∞} values were significantly higher, prior to the removals, for most Ritchie Bay plots, (i.e., Plot 1 higher in 1991 than 1995 Plot 1 in 1995 and 2000 were not significantly different), Plot 2 and 4 larger in 1995 than 2000, Plot 3 larger in 1992 than for 2000, and Marina Island Plot 3 larger for 1992 than for 2001. In contrast, the L_{∞} values for Ritchie Bay Plot 4 were not significantly different between 1995 and 2000.

TABLE 4.

Density estimate of geoduck recruitment to harvestable size in experimental plots of Ritchie Bay and Marina Island.

		Age (year)	Number of geoduck m ⁻²		
Year	Plot		Mean	Lower 95% CI	Upper 95% CI
Ritchie Bay					
1991	1	6	0.104	0.053	0.179
1992	3	6	0.021	0.006	0.039
1995	1	6	0.068	0.032	0.114
1995	2	6	0.051	0.000	0.096
1995	4	6	0.109	0.055	0.169
2000	1	6	0.177	0.122	0.231
2000	2	6	0.179	0.095	0.285
2000	3	6	0.165	0.127	0.203
2000	4	6	0.137	0.089	0.192
Marina Island					
1992	3	7	0.028	0.017	0.043
2001	1	7	0.009	0.005	0.014
2001	2	7	0.020	0.013	0.029
2001	3	7	0.030	0.021	0.041

CI, confidence interval.

Between Plots Within Similar Years

The k value(s) for geoduck in Ritchie Bay Plot 1 in 1991 was not significantly different to Plot 3 in 1992; for 1995 Plots 1, 2 and 4 were not significantly different; for 2000 Plots 1, 2, and 4 were not significantly different but all three were significantly lower than that for Plot 3. The k values were not significantly different for Marina Island Plots 1, 2, and 3 in 2001. The k value was higher for Marina Island Plot 3 in 1992 than for Ritchie Bay Plot 1 in 1991 and Plot 3 in 1992.

The L_{∞} values were significantly different between most

TABLE 5.

Density of geoduck from the sampled Ritchie Bay study plots to show the possible influence on recruitment of 4-year-olds in 1995 and 9-year-olds in 2000 by artificial seeding in 1991.

Year	Plot	Age (year)	Number of Geoduck m^{-2}		
			Mean	Lower 95% CI	Upper 95% CI
1995	1*	4	0.60	0.47	0.74
1995	2*	4	0.33	0.21	0.48
1995	4	4	0.14	0.08	0.20
2000	1*	9	0.19	0.14	0.25
2000	2*	9	0.24	0.14	0.36
2000	3	9	0.07	0.05	0.10
2000	4	9	0.10	0.06	0.14

* Plots 1 and 2 were seeded with hatchery raised geoduck juveniles (≤ 0.6 year) during September 1991. Plots 3 and 4 were not artificially seeded. CI = confidence interval.

TABLE 6.

von Bertalanffy growth parameters for *P. abrupta* from experimental plots at Ritchie Bay and Marina Island.

Plot	Year	L_{∞}	k	σ^2	n
Ritchie Bay					
1	1991	150.5 bex (146.3–155.7)	0.234 a (0.204–0.269)	16.62 (14.48–19.54)	82
1	1995	140.4 a (139.2–141.9)	0.475 b (0.432–0.521)	10.54 (9.93–11.49)	314
1	2000	139.9 a (137.5–141.6)	0.319 c (0.294–0.352)	13.48 (12.59–14.59)	427
2	1995	150.5 c (149.5–151.7)	0.405 d (0.372–0.437)	10.81 (10.06–11.60)	397
2	2000	148.5 b (147.2–149.8)	0.304 c (0.282–0.335)	10.95 (10.22–11.94)	365
3	1992	159.0 (151.1–164.8)	0.236 a (0.217–0.266)	16.36 (13.98–20.61)	58
3	2000	144.3 (142.2–146.4)	0.413 db (0.382–0.461)	9.96 (9.18–10.94)	297
4	1995	147.6 b (146.3–148.9)	0.370 d (0.339–0.412)	11.62 (10.76–12.75)	365
4	2000	148.0 b (146.8–149.3)	0.306 c (0.285–0.342)	10.37 (9.74–11.19)	421
1,2,3,4	2000	145.8 (145.0–146.8)	0.328 (0.314–0.340)	12.14 (11.64–12.64)	1510
Marina Island					
1	2001	137.8 (136.5–139.2)	0.277 x (0.259–0.300)	10.78 (9.95–11.61)	348
2	2001	134.4 m (132.5–136.5)	0.262 x (0.245–0.284)	11.57 (10.53–12.91)	223
3	1992	149.6 x (144.7–155.9)	0.155 (0.136–0.172)	19.18 (17.21–22.03)	90
3	2001	131.0 m (129.0–133.1)	0.266 x (0.250–0.285)	10.82 (9.93–11.99)	255
1,2,3	2001	136.1 (135.0–137.1)	0.252 (0.244–0.263)	11.35 (10.84–12.03)	826

Values in brackets are approximate 95% confidence intervals. The von Bertalanffy parameter t_0 was set at zero. Mean L_{∞} and k values followed by the same letter and column are not significantly different ($P > 0.05$); whereas values not followed by the same letter are significantly different ($P < 0.05$) using the Kimura method (see text and Table 6 for more details). Comparisons of geoduck growth in plots between study areas were conducted only for plots that were sampled in the same year or one year apart. σ^2 is the mean square error of the model.

Ritchie Bay plots during 1995 and during 2000, except Plot 2 and 4 were not significantly different during 2000. The L_{∞} values for 2001 at Marina Island Plots 2 and 3 were not significantly different, but both were different from Plot 1. The L_{∞} value for Marina Island Plot 3 in 1992 was not significantly different for Ritchie Bay Plot 1 in 1991, but significantly different for Plot 3 in 1992.

Between Plots and Years

The k values were significantly different when comparing between most plots and years, except for Ritchie Bay Plots 1, 2, and 4 in 1995, which were similar to Plot 3 in 2000.

The L_{∞} values were not significantly different between Ritchie Bay Plot 1 in 1991 with Plot 2 and 4 in 1995, with Plots 2 and 4 in 2000, and Plot 4 in 1995 with Plot 2 in 2000.

Both k and L_{∞} geoduck growth values were significantly higher for the combined Ritchie Bay Plots 1, 2, 3, and 4 data in 2000 compared with the combined Marina Island Plots 1, 2, and 3 data in 2001.

Relation Between Density, Recruitment, and k

No significant ($P > 0.05$) relationships between recruitment and density, k and recruitment, or k and density could be detected when using data from all plots and years in the Ritchie Bay or the Marina Island study areas.

DISCUSSION

This study provides, for the first time, information on *P. abrupta* population biology from a 9-year study of experimental plots in two different areas of southern BC. Heavy exploitation for the first 3 years in Plot 3 of both Ritchie Bay and Marina Island substantially reduced geoduck population densities, reduced the average age (by removing old individuals), but thereafter densities slowly increased due to recruitment. A similar trend was observed for geoduck that were fished heavily only for the first year, but the

initial recovery was more rapid in geoduck populations for Plot 1 of Ritchie Bay than for Plot 2 of Marina Island. Geoduck densities in the control plots fluctuated over time, but CIs often overlapped between years. Factors causing geoduck age densities to fluctuate could have included removals for biosamples, fishery induced mortality (harvesting methods causing incidental injuries to the remaining unfished geoduck), geoduck natural mortality (e.g., Zhang & Campbell 2004), recruitment and growth rates influenced by environmental conditions. The fluctuation was also likely related to the accuracy of the density estimates. Although experienced commercial divers were used for all density estimates, occasional poor visibility, due to plankton, drift algae, silt, or strong current at Ritchie Bay could have influenced the results.

Geoduck densities surveyed in the experimental plots were generally within the density range reported from surveys in similar regions: west coast of Vancouver Island ($0.29\text{--}3.34\text{ m}^{-2}$) (Hand & Dovey 1999, Hand & Bureau 2000) and Inside waters including Georgia Strait ($0.18\text{--}0.70\text{ m}^{-2}$) (Hand & Bureau 2000). The high density values of $5\text{--}7\text{ m}^{-2}$ found in Plot 2, and generally from Ritchie Bay by Fyfe (1984) ($4.00\text{--}7.69\text{ m}^{-2}$), were unusual for southern BC and only matched and surpassed by areas surveyed in northern BC with densities as high as 10.27 m^{-2} found in the Moore Islands (Hand & Bureau 2000). A reason for the high geoduck density at Ritchie Bay was probably because of the area being closed to commercial fishing and designated as a research reserve since 1980 (Fyfe 1984). In contrast, the area around Marina Island was heavily harvested during 1978–1989 and closed to commercial fishing since 1990 (Campbell et al. 1996b).

Geoduck percentage shows were high ($>80\%$) in both study areas for most of the summers studied. Low show factors ($<80\%$) recorded in the Marina study area during 1993–1994 were preceded by strong winds of $28\text{--}30\text{ km per hour}$ a few days prior to the density surveys. High winds probably caused the geoduck to retract their siphons via shifting sand or debris on the substrate surface at the study sites where depths were fairly shallow (<17

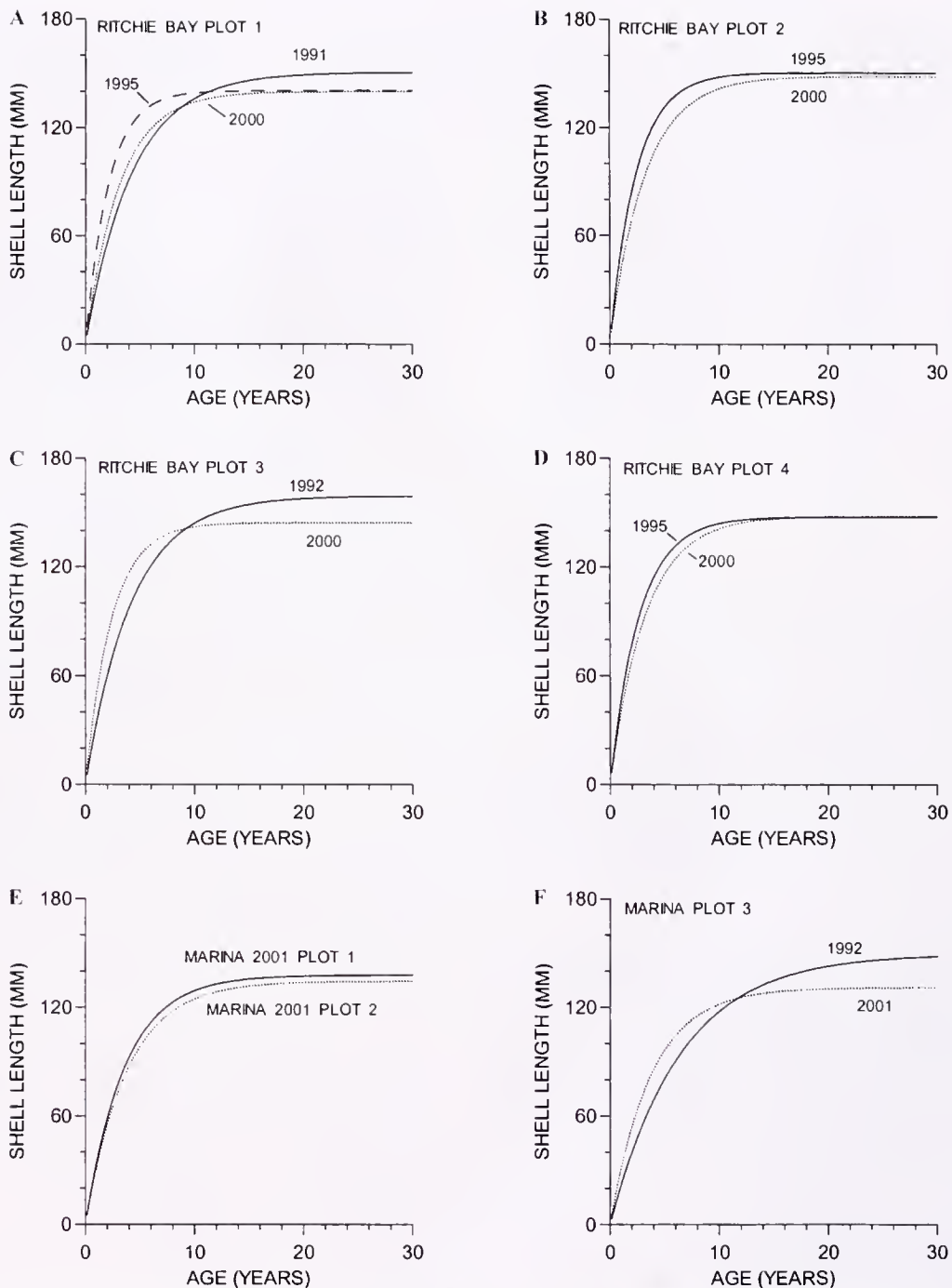


Figure 5. Mean geoduck growth curves from the experimental plots of Ritchie Bay and Marina Island derived from equation coefficients in Table 6. Curves shown are for only geoduck ≤ 30 y old to clearly show the rapid growth increase to the asymptotic shell length.

m). Variation in the direction, duration, and/or intensity of wind may have influenced the level of geoduck show factor in different locations, depending on topography and depth of the area. Low salinity caused by precipitation and rainwater runoff from nearby land due to storms may also be other factors affecting geoduck shows. Show factor estimates could be important in estimating density, especially when density surveys are conducted after a storm or during fall-winter months. The percent of geoduck in a bed "showing" their siphons at or above the substrate surface has been found in different studies to vary under different environ-

mental and physiologic conditions. A number of authors (Goodwin 1973, Goodwin 1977, Cox & Charman 1980, Turner & Cox 1981, Fyfe 1984) have reported that the highest seasonal percentage of shows ($>80\%$) was during the summer (April to September) when feeding and reproduction occurred and the lowest ($<50\%$) was during the winter (November to February) when many geoduck were inactive. Shows were highest when local water currents were not excessive and there was no mechanical disturbance of the bottom (Goodwin 1977). Campbell et al. (1996a) documented the percentage shows was reduced from about 80% to 65% after a

storm in a wave-exposed area, but returned to about 80% within 2–3 wk of the storm event.

The recruit age for geoduck was lower for Ritchie Bay (6 y) than for Marina Island (7 y) because geoduck growth rates were highest in Ritchie Bay of the two study areas. This suggests that growth rates, physical or physiologic characteristics and age estimation for geoduck from different locations need to be examined prior to defining an age for a particular type of recruitment, (e.g., neck size for fishery purposes or for age at maturity, Campbell & Ming 2003).

Recruitment was variable temporally and at both small and large spatial scales. Recruitment was generally higher for the Ritchie Bay plots during 2000 and for Marina Island Plot 3 during 2001 than the previous years. Recruitment was considerably higher for geoduck in Ritchie Bay during 2000 than for Marina Island during 2001. Recruitment was similar for Ritchie Bay plots with high or low geoduck densities during 2000. Spatial and temporal variability in geoduck recruitment have been reported previously (Breen & Shields 1983, Harbo et al. 1983, Fyfe 1984, Goodwin & Shaul 1984, Sloan & Robinson 1984, Breen et al. 1991, Noakes & Campbell 1992, Hand & Dovey 1999, Orensanz et al. 2000, Bureau et al. 2002, Bureau et al. 2003). Recruitment may be influenced by various factors (e.g., size of breeding brood stock providing progeny, predation on the larval and early juvenile stages, environmental variables such as local currents, and/or general temperature trends affecting larval and early juvenile growth and survival). The timing of reproduction may also affect larval settlement and juvenile recruitment through food availability and growth rates. Gribben et al. (2004) found that differences in the timing of spawning between two *P. zelandica* populations could be attributed to latitudinal temperature gradients. Bureau et al. (2002, 2003) found that age frequency data of geoduck sampled throughout BC during the 1993 to 2002 period indicated strong geoduck recruitment trends, which might be linked to a general warming trend or El Niño events.

Local geoduck recruitment also could be affected by the physical disturbances of local fishing events. The study of Goodwin and Shaul (1984), which lasted approximately 20 mo and sampled >1 year-old geoduck with a venture dredge, suggested that fishing could have an adverse effect on recruitment in the short term. Our 9-y study examined the long term side effects of fishing on recruitment, since young geoduck (<4 y) could not be detected until the survivors recruited into 6–7 y age groups and became visible to divers several years following the fishing event. In this study, we did not detect any long-term effect of heavy fishing or low geoduck densities on subsequent recruitment. However, Zhang and Campbell (2004) found from retrospective age structure modeling of these populations that recruitment was generally lower after fishing geoduck in Plot 3 compared with the other three plots from Ritchie Bay suggesting that fishing, low geoduck densities or juvenile mortality could have influenced recruitment in Plot 3, at least in the first 1–3 y after fishing. Assuming there was sufficient geoduck larval supply from other areas, we concluded (this study and Zhang & Campbell 2004) that a heavy fishing event could reduce local recruitment in the short term, but probably not influence recruitment in the long term (e.g., >3 y). However, other environmental or biologic factors could mask the effects of fishing on recruitment as was observed in the Marina Island study area. In contrast to the Ritchie Bay observations, at Marina Island, the control unfished Plot 1 had poor recruitment compared with the other two fished plots.

TABLE 7.

Results of the likelihood ratio tests using the Kimura (1980) method to compare von Bertalanffy growth parameters of geoduck between years and plots for Ritchie Bay and Marina Island.

Comparisons				P-values for Hypotheses		
Plot	Year versus	Plot	Year	H1	H2	H3
Ritchie Bay						
1	1991	1	1995	0	0	0
1	1991	1	2000	0	0	0
1	1991	2	1995	0	1.0	0
1	1991	2	2000	0	0.30	0
1	1991	3	1992	0.88	0.02	0.03
1	1991	3	2000	0	0	0
1	1991	4	1995	0	0.13	0
1	1991	4	2000	0	0.16	0
1	1995	1	2000	0	0.65	0
1	1995	2	1995	0.049	0	0
1	1995	2	2000	0	0	0
1	1995	3	1992	0	0	0
1	1995	3	2000	0.06	0	0
1	1995	4	1995	0	0	0
1	1995	4	2000	0	0	0
1	2000	2	1995	0	0	0
1	2000	2	2000	0.44	0	0
1	2000	3	1992	0	0	0
1	2000	3	2000	0	0	0
1	2000	4	1995	0.04	0	0
1	2000	4	2000	0.44	0	0
2	1995	2	2000	0	0.02	0
2	1995	3	1992	0	0	0
2	1995	3	2000	0.76	0	0
2	1995	4	1995	0.26	0	0
2	1995	4	2000	0	0	0
2	2000	3	1992	0	0	0
2	2000	3	2000	0	0	0
2	2000	4	1995	0	0.31	0.03
2	2000	4	2000	0.84	0.57	0.83
3	1992	3	2000	0	0	0
3	1992	4	1995	0	0	0
3	1992	4	2000	0	0	0
3	2000	4	1995	0.13	0.01	0.04
3	2000	4	2000	0	0	0
4	1995	4	2000	0	0.61	0.01
Marina Island						
1	2001	2	2001	0.30	0.02	0
1	2001	3	1992	0	0	0
1	2001	3	2001	0.42	0	0
2	2001	3	1992	0	0	0
2	2001	3	2001	0.70	0.08	0.03
3	1992	3	2001	0	0	0
Ritchie Bay						
1	1991	3	1992	0	0.81	0
3	1992	3	1992	0	0.02	0
1,2,3,4	2000	1,2,3	2001	0	0	0

Hypotheses: H1: Common k , i.e., $L_{\infty}(1) \neq L_{\infty}(2)$ and $k(1) = k(2)$; H2: Common L_{∞} , i.e., $L_{\infty}(1) = L_{\infty}(2)$ and $k(1) \neq k(2)$; H3: Common k and L_{∞} , i.e., $L_{\infty}(1) = L_{\infty}(2)$ and $k(1) = k(2)$. The null hypothesis rejected at $\alpha < 0.05$ level. P-values of 0 are <0.005.

Artificial seeding of Ritchie Bay Plots 1 and 2 provided a slight increase above the natural recruitment. Much (96% to 99%) of the large number of seed released (estimated to be a mean 16.0 m^{-2}) into these two plots probably did not survive. Without providing adequate protection from predators (e.g., fish, crabs, and starfish) (Goodwin & Pease 1989) artificial geoduck seeding was considered not economically feasible for enhancement of natural geoduck populations (Beattie 1992, Beattie & Blake 1999, Clapp 2000).

Growth characteristics of geoduck from different plots and years varied considerably. Although no simple broad statistical relationship could be detected between geoduck density and the growth parameters when all plots and years for each study area were combined some interesting observations on geoduck growth could be made. Following removal of many geoduck in a plot, subsequent growth characteristics indicated a lowering of the apparent mean max asymptotic length (L_{∞}) and an increase in the Brody growth coefficient (k) of the von Bertalanffy growth model. Removal of old individuals from a geoduck population such as in Ritchie Bay Plots 1 and 3 and Marina Island Plot 3 (Fig. 1, 3, and Table 3) probably influenced the L_{∞} to decline and the Brody growth function k values to increase (Fig. 5 and Table 6). Where there was no major exploitation and geoduck densities remained similar over time such as in Ritchie Bay Plots 2 and 4, L_{∞} values remained similar, but k values varied with time. Reasons for the differences in *P. abrupta* growth rates between areas and plots could be generally attributed to various environmental and bio-

logic factors associated with different habitats (e.g., depth, substrate type, temperature, exposure to water surge activity, pollution, food availability, geoduck density, and or genetic characteristics) (Breen & Shields 1983, Harbo et al. 1983, Goodwin & Shaul 1984, Goodwin & Pease 1991, Noakes & Campbell 1992, Hoffmann et al. 2000, Bureau et al. 2002).

ACKNOWLEDGMENTS

The authors thank D. Brouwer, D. Bureau, W. Carolsfeld, B. Clapp, J. Clarke, L. Convey, R. Khan, C. Hand, R. Harbo, S. Heizer, L. Lee, J. McArthur, F. Merilees, J. Nash, J. Rogers, T. Scott, K. Southey, N. W. Surry, W. Thompson; and T. White for technical assistance; W. Hajas for statistical advice; many geoduck commercial fishermen: R. Antifave, M. Barcelonne, R. Barton, M. Boudreau, K. Brewer, P. Boulton, B. Crawford, G. Hache, R. Hais, G. Hickie, J. Hume, T. Keith, R. Kowatski, D. Larson, J. M. LeGuerrier, M. Lanoie, G. MacDonald, G. McKay, K. Montgomery, D. Mousseau, M. Renshaw, S. Renshaw, A. Schofield, T. Somerville, G. Sorensen and N. Sorenson, S. Soucy, R. Sutherland, K. Tull, L. Tulloch, B. Wallace-Tarry; Underwater Harvesters Association, for assisting with the field experiments; Pelegos Trucking and CF McLean Trucking for transporting samples and shells; J. H. Beattie, Washington Department of Fish and Wildlife, for supplying the hatchery reared juvenile geoduck; C. Hand, W. Hajas and anonymous reviewers for helpful comments, which improved this manuscript.

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NATURAL MORTALITY AND RECRUITMENT RATES OF THE PACIFIC GEODUCK CLAM (*PANOPEA ABRUPTA*) IN EXPERIMENTAL PLOTS

ZANE ZHANG AND ALAN CAMPBELL*

Shellfish Stock Assessment Section, Pacific Biological Station, 3190 Hammond Bay Road, Nanaimo, British Columbia, V9T 6N7, Canada

ABSTRACT Experiments were conducted in Ritchie Bay on the west coast of Vancouver Island and Marina Island inside the Strait of Georgia, British Columbia, to examine the natural mortality (M) of geoduck, *Panopea abrupta*, and impact of harvesting on recruitment to 1 year of age. We used age-structured models to estimate M and recruitment rates. The overall mean M was estimated to be 0.039 (0.0046 SE) using Monte Carlo simulations and to be 0.036 (0.0027 SE) using the Bayesian method. Severe harvesting (>90% removal) had an adverse impact on short-term (≤ 3 y) recruitment, but did not show evidence of long-term (>3 y) impact on geoduck recruitment in the Ritchie Bay experimental plots. In contrast, the most severely harvested plot of the Marina Island plots received the highest recruitment both in the short-term and long-term. However, this plot also obtained the highest recruitment before the experiment, suggesting that local hydrodynamics may have played a significant role in the geoduck larval settlement / recruitment rates in the Marina Island study area.

KEY WORDS: bivalvia, geoduck, *Panopea abrupta*, modeling, natural mortality, recruitment, larval settlement

INTRODUCTION

The Pacific geoduck clam, *Panopea abrupta*, (Conrad 1849) (Bivalvia: Hiatellidae), is found in subtidal coastal areas along the northeast Pacific from California to Alaska (Coan et al. 2000). *Panopea abrupta* supports commercial fisheries in Washington State, British Columbia (B.C.) and Alaska (Campbell et al. 1998, Bradbury & Tagart 2000). In an attempt to understand the effects of fishing on the population biology of geoduck, Campbell et al. (2004) followed geoduck population density, recruitment, and growth under different controlled exploitation levels in experimental plots of southern B.C. over a 9-y period. In our study we extend the analyses of Campbell et al. (2004) by using a retrospective age structured model to determine natural mortality and recruitment rates of geoduck in these plots. Campbell et al. (2004) defined recruitment rate as the density of age 6 or 7 y geoduck growing into the population that was fully vulnerable to fishing. In this study, however, we define recruitment rate as the number of age 1-y geoduck per m^2 , to obtain a few more years of density data for young geoduck that settled after the beginning of the experiment. Unless specified, recruitment refers to 1-year-old geoduck. Understanding natural mortality and impact of harvesting on recruitment are important to managers in establishing sustainable harvest rates or annual fishing quotas for geoduck fisheries (Bradbury & Tagart 2000, Hand & Bureau 2000, Orensanz et al. 2000).

MATERIALS AND METHODS

Two experiments were conducted, one in Ritchie Bay on the west coast of Vancouver Island and the other in Marina Island inside the Strait of Georgia, B.C. The experiment started in 1991 and ended in 2000 in Ritchie Bay with four experimental plots; the first three had an area of 2440 m^2 each, and the fourth was 2500 m^2 . The experiment started in 1992 and ended in 2001 in Marina Island with all three experimental plots having an area of 2500 m^2 each. Because details of the study areas can be found in Campbell et al. (2004), we only summarize the relevant field procedures.

The general harvest treatment in each plot for Ritchie Bay was

(1) moderate, (2) none, (3) heavy, and (4) none (control), and for Marina Island was (1) none (control), (2) moderate, and (3) heavy. A large number of geoduck were removed commercially in the first experimental year from Plot 1 and in the first 3 y from Plot 3, whereas no geoduck were removed commercially from Plot 2 or 4 in Ritchie Bay. In Marina Island, geoduck were removed commercially in the first experimental year from Plot 2 and in the first 3 y from plot 3, whereas no geoduck were removed commercially from Plot 1. In addition, about 500 geoduck were removed as bio-samples each time in 2 to 3 different years from each of the four plots in Ritchie Bay. In Marina Island, bio-samples of about 300 to 400 geoduck were also removed from each of the three plots during 2001, and about 500 geoduck were also taken from Plot 3 during 1992. The bio-samples were used to determine geoduck age frequencies. Age of each geoduck was estimated using the acetate peel method of Shaul and Goodwin (1982).

In most of the experimental years, dive surveys were conducted to assess the abundance of geoduck in each plot in both experimental areas. Density surveys were always conducted prior to removal of geoduck. All geoduck densities were adjusted for show factor (a detectability proportion) as provided by Campbell et al. (2004). To determine if seeding of hatchery-raised juvenile geoduck could supplement natural recruitment, Campbell et al. (2004) artificially seeded plot 1 and plot 2 of Ritchie Bay, with <0.5-y-old hatchery-raised juvenile geoduck during September 1991.

Age-structured modeling was used to estimate natural mortality (M) and recruitment rates. We first estimated vulnerabilities of young geoduck to the divers to estimate abundance of young geoduck using the catch data. We estimated M by comparing model-derived abundances with survey-derived abundances. Using the calculated M s and available bio-samples, we estimated 1-y-old geoduck densities during and 1–10 y prior to the experiment. Mortality rate for geoduck was considered to be high during the early benthic life stage (Goodwin & Shaul 1984), and decreased quickly after age 1 year (Sloan & Robinson 1984). Thus, the density of 1-y-old geoduck was used to denote the recruitment rate and to reflect the relative larval settlement rate of the previous year.

Vulnerability to Fishing

One-year-old Geoduck did not appear to be vulnerable to divers, as only 1 out of 4354 aged geoduck was estimated to be age

*Corresponding author. E-mail: ZhangZ@pac.dfo-mpo.gc.ca

1 y. We assumed that geoduck started to be partially vulnerable at age 2 y and fully vulnerable at age 6 y in Ritchie Bay and 7 y in Marina Island (Campbell et al. 2004). We used an exponential model to describe the vulnerability of geoduck between ages 2 y and fully vulnerable age (FV_α) of ages 6 y or 7 y:

$$V_\alpha = \frac{(\alpha - 1) \times \exp(\beta \times \alpha)}{(FV_\alpha - 1) \times \exp(\beta \times FV_\alpha)} \quad (1)$$

where α is the age and β is the parameter of the model. The value of β is estimated by fitting a multinomial probability model to the observed age frequencies. The likelihood for observing the age composition in a bio-sampling occasion was estimated based on the age composition from the previous bio-sampling occasion and vulnerability of geoduck at each age. Only geoduck between ages 2 and FV_α years in one sampling occasion were used to calculate the probabilities for age composition for geoduck between $2 + n$ and $FV_\alpha + n$ years in the next sampling occasion n years later. Geoduck $>FV_\alpha$ were not considered, because the likelihood for observing the age composition for geoduck $>FV_\alpha + n$ in the next sampling occasion was not affected by different values of β .

Only age-composition data from Plots 1, 2, and 4 in Ritchie Bay were used for the vulnerability estimation; data from Plot 3 in Ritchie Bay were not used because they would have been affected by commercial removal of geoduck during two sampling occasions, and there was an insufficient number of bio-samples over time from Marina Island.

The density of geoduck at age α ($2 \leq \alpha \leq FV_\alpha$) was estimated to be:

$$D_{j,\alpha} = \frac{\hat{D}_j \times p_{j,\alpha}}{V_\alpha} \quad (2)$$

where \hat{D}_j and $p_{j,\alpha}$ are the survey-derived geoduck density and the proportion of geoduck of age α in bio sampling occasion j .

In the next bio-sampling occasion n years later, the surviving geoduck of age α would become age $\alpha + n$. We assumed that natural mortality rate was invariant with ages. Thus, the proportion of vulnerable geoduck at age $\alpha + n$ in bio-sampling occasion $j + 1$ was estimated to be:

$$p_{j+1,\alpha+n} = \frac{D_{j,\alpha} \times V_{\alpha+n}}{\sum_{\alpha=2}^{FV_\alpha} D_{j,\alpha} \times V_{\alpha+n}} \quad (3)$$

The estimated proportions were treated as probabilities for the multinomial distribution. The likelihood for observing the age composition at the bio-sampling occasion $j + 1$ was calculated as:

$$L_{j+1} = \prod_{\alpha=2}^{FV_{\alpha+n}} (p_{j+1,\alpha+n})^{N_{j+1,\alpha+n}} \quad (4)$$

where $N_{j+1,\alpha+n}$ is the observed number of geoduck at age $\alpha + n$ at bio-sampling occasion $j + 1$. The overall likelihood for observing the age compositions in all bio-sampling occasions except for the first ones in all the plots was:

$$L = \prod_{Plots} \prod_j L_{j+1}$$

The unknown parameter β in Eq. 1 was estimated by trials of various values until the maximum likelihood was reached.

To assess the variation in estimated parameter β , age composition in each of the bio-samples was bootstrapped by resampling

with replacement to produce a simulated sample of the same size. The simulated data sets were used to estimate the parameter β in the same way as described earlier. Nine hundred and ninety-nine data sets were generated producing 1000 estimates of β , from which the mean and standard error for β were calculated.

Natural Mortality Rate

We reconstructed population age structure using the bio-samples in the last experimental year. M was estimated to be the value that resulted in the best match between the model-derived abundances and the survey-derived abundances. We estimated variations in M with Monte Carlo simulations (i.e., age composition, density, and the value of β) of the vulnerability model are randomly regenerated. For a comparison, the Bayesian method (Carlin & Louis 1996) was also used to estimate M .

The minimum age was set at 2 y for each year. The maximum age was set to be 30 y for the last experimental year. The maximum age for other years was 1 year younger than for the subsequent year. Geoduck older than the maximum age were pooled together to form a maximum plus age group. For instance, geoduck older than age 30 y for the last year were lumped to be age 30 + y. In the mathematical equations, the pooled age group was expressed as an age 1 year older than the maximum age considered. For instance, age 31 y for the last year means age 30 + y in the expression.

The number of geoduck at age α in the last bio-sampling year ($y = 2000$ for Ritchie Bay and $y = 2001$ for Marina Island) was estimated based on the survey-derived density (\hat{D}_y), area of the plot (S), the proportion of geoduck at age α in the samples ($p_{y,\alpha}$), and vulnerability of geoduck at age α (V_α):

$$N_{y,\alpha} = \hat{D}_y \times S \times p_{y,\alpha} / V_\alpha \quad (5)$$

To reconstruct age compositions in the past, we replaced the amount of geoduck removed commercially and/or due to bio sampling back to the reconstructing population. We used the proportion of geoduck at each age in the bio-samples to estimate the amount of removal at each age, if bio-samples were taken in that year. Otherwise, the proportion was estimated based on the reconstructed age composition in the subsequent year. The proportion of vulnerable geoduck at age α in year y was:

$$\begin{cases} VP_{y,\alpha} = p_{y,\alpha} & \text{with bio-samples in year } y \\ VP_{y,\alpha} = \frac{N_{y+1,\alpha+1} \times V_\alpha}{\sum_{\alpha=2}^{MA_{y+1}} N_{y+1,\alpha+1} \times V_\alpha} & \text{without bio-samples in year } y \end{cases} \quad (6)$$

where MA_{y+1} is the maximum age considered for year $y + 1$. The number of geoduck of age α removed in year y was estimated to be:

$$RM_{y,\alpha} = VP_{y,\alpha} \times TR_y \quad (7)$$

where TR_y is the total number of geoduck removed in year y . The number of geoduck at age α in year y ($y < \text{last experimental year}$) was back-calculated from the estimated number of geoduck at age $\alpha + 1$ in year $y + 1$:

$$N_{y,\alpha} = N_{y+1,\alpha+1} \times \exp(M) + RM_{y,\alpha} \quad (8)$$

where M is the natural mortality rate to be estimated in the model. Eq. 6–8 were repeatedly used to estimate abundance of geoduck at each age in each of the previous years.

The likelihood for the reconstructed population was determined by comparing the model-estimated abundances with the survey-estimated abundances. Because abundances derived from surveys indicated the vulnerable population abundances, the likelihood was calculated as follows:

$$L = \frac{1}{se_v} \exp \left(- \frac{\sum_y (VN_y - \hat{N}_y)^2}{2(se_v)^2} \right) \quad (9)$$

where N_y and se_v are, respectively, the number of geoduck and standard error estimated from the survey, and VN_y is the model-estimated vulnerable number of geoduck in year y when bio-samples were taken:

$$VN_y = \sum_{\alpha=2}^{MA_y+1} N_{y,\alpha} \times V_{\alpha} \quad (10)$$

When data from all the plots were analyzed simultaneously, the likelihood was simply the product of likelihood for each of the plots:

$$LL = \prod_{Plots} L.$$

Monte Carlo Simulation

A grid search method was used to find the value of M , which maximized the likelihood. Errors in the estimations were evaluated through simulations. Age composition in the bio-samples taken in the last year was bootstrapped (Efron & Tibshirani 1993) by re-sampling with replacement to produce a simulated sample of the same size. Geoduck densities in the last year were randomly regenerated from a normal distribution with the mean and standard error estimated based on the survey. The parameter β in the vulnerability model was also randomly regenerated from a normal distribution with the estimated mean and standard error. The calculation procedures described earlier were repeated for each set of regenerated data. Nine hundred and ninety-nine data sets were regenerated, resulting in 1000 sets of estimates of M .

Bayesian Analysis

The parameters in the Bayesian model include M , β , and density or densities in the last bio-sampling year (one for each plot). Uniform prior probability distributions were applied to all the parameters. The range was constrained between 0 and 0.3 for M and larger than 0 for densities. In the case that only one plot is used in the estimation, the posterior probability could be expressed as follows:

$$P \propto L \times \frac{1}{SEd} \exp \left(- \frac{(D - \hat{D})^2}{2 \times SEd^2} \right) \times \frac{1}{SEb} \exp \left(- \frac{(\beta - \hat{\beta})^2}{2 \times SEb^2} \right) \quad (11)$$

where L is the likelihood calculated using Eq. 9; D , \hat{D} and SEd are, respectively, the regenerated true density, survey-estimated density, and standard error in the last bio-sampling year; β , $\hat{\beta}$ and SEb are, respectively, the regenerated true value, estimated value for the parameter of the vulnerability model and associated standard error. When all plots were used simultaneously, the posterior probability was:

$$PP = \prod_{Plots} P.$$

The Gibbs sampler (Geman & Geman 1984) was used to generate marginal likelihood distribution for M . The *Gibbs* sampler is an approach for sequentially taking random samples for each parameter from its full conditional distribution, which is a probability distribution conditional on the data and on the current values of all the other parameters (Gelfand & Smith 1990). To construct the full conditional distribution for a parameter, we just picked the terms in the posterior distribution which involve the parameter (Gilks et al. 1996). Thus, the full conditional distribution is proportional to the posterior probability. When a full conditional distribution can be expressed in a special form, sampling can be easily conducted. The full conditional distributions for M , D and β , however, cannot be expressed as some special forms. We used the Adaptive Rejection Metropolis Sampling algorithm developed by Gilks et al. (1995) to take random samples.

To start the Gibbs sampler, the initial values for M , densities, and β were set to be the estimated means using the Monte Carlo simulation method described earlier. Samples of the first 500 cycles were ignored as a "burn-in" period for the initial algorithm setup. Altogether, 20,500 cycles were conducted. To reduce autocorrelation every 20th sample was saved after the burn-in period. The MCMC samples passed the convergence test of Heidelberger and Welch (1983) provided by the computer program CODA (Convergence Diagnosis and Output Analysis Software for Gibbs sampling output version 0.40) (Best et al. 1997).

The Bayesian method described earlier assumed that the estimated age for each sample represents the true age with no errors. However, age composition in the bio-samples taken in the last year was bootstrapped, when the Monte Carlo method was used to estimate the variation in M . For a more comparable comparison of the estimated variations in M between the two methods, age composition in the bio-samples collected in the last year was also bootstrapped after each random sampling of M , β , and density or densities.

Recruitment

To compare recruitment rates before and after the beginning of the experiment, population age structures were constructed from the last experimental year back to 10 y before the beginning of the experiment for each plot. Because bio-samples collected in earlier years were likely to be of higher significance in determining recruitment before the experiment, bio-samples collected in all the years were independently used in the reconstruction. Both the estimated M for the individual plot and overall mean M using the Bayesian method with bootstrap of the age composition were separately applied for Ritchie Bay data. Only the overall mean M estimated for geoduck in Ritchie Bay was used for each plot in Marina Island, because natural mortality rates could not be reliably estimated for geoduck in each experimental plot of Marina Island.

The maximum and minimum ages considered for each year were the same as described earlier. The minimum age was age 2 y for each year; the maximum age was age 30 y for the last experimental year, and the maximum age for other years was just 1 year younger than for the subsequent year. The reconstruction was also conducted analogously as described earlier.

Multiple estimates of the number of 1-y-old geoduck were obtained for each year, when there were bio-samples from multiple bio-sampling years used for the reconstruction. The number of

1-y-old geoduck in each year was calculated as a weighted mean of the multiple estimates:

$$R_y = \frac{\sum_t R_{t,y} \times W_{t,y}}{\sum_t W_{t,y}} \quad (12)$$

where $R_{t,y}$ is the estimated number of 1-y-old geoduck in year y based on the bio-samples collected in year t , and $W_{t,y}$ is the weight defined to be negatively correlated with the time interval between y and t :

$$W_{t,y} = \frac{1}{t - y + 1}$$

Simulations were conducted to generate confidence intervals for the estimates. Age compositions of the bio-samples, population densities in the bio-sampling years, and β of the vulnerability model were randomly regenerated in the way as described. In addition, M was also randomly regenerated from a normal distribution with the mean and standard error set to be the estimated ones using the Bayesian method with bootstrap of the age composition. The calculations were repeated for each set of regenerated data. A total of 999 data sets was regenerated, resulting in 1000 sets of population reconstructions.

RESULTS

Vulnerability

The parameter, β , in the vulnerability model (Eq. 1) was estimated to be 0.357 (0.124 SE) or 0.144 (0.099 SE) when age 6-y or 7-y geoduck, respectively, were assumed to be fully vulnerable to the fishery.

Natural Mortality

Estimated variations in M by the Monte Carlo method or the Bayesian method for different plots in Ritchie Bay appeared to be similar (Table 1). Geoduck in Plot 1 and 2 had the highest and lowest M , respectively, and geoduck in Plot 4 had a higher M than those in Plot 3. Estimated overall mean M was slightly higher using the Monte Carlo method than that using the Bayesian method (see Table 1). The overall mean M estimate was 0.039 (11.8% coefficient of variation, CV) using the Monte Carlo method, and 0.036 (7.5% CV) using the Bayesian method with bootstrap of the age composition. The estimated mean values of M were almost identical, whether bootstrap was applied on the age composition or not. Variances of M values were higher with the bootstrapping than without the bootstrapping.

Values of M for geoduck in Marina Island could not be reliably estimated. Using the Monte Carlo method, the estimated mean M was 0.06 for Plot 1, but the SE was 0.033 with a CV of 53.3% (see Table 1). For Plot 2, estimated M values were negative in approximately 50% of the simulations. For Plot 3, estimated M values were negative in all the simulations.

Recruitment

The general patterns of recruitment rates were similar among individual beds in Ritchie Bay, whether the estimated M for individual beds or overall mean M was used. Therefore, we only reported our findings using the overall mean M .

During the 10 y before the experiment and in the 1st experimental year of 1991 at Ritchie Bay, recruitment rates were similar among the four plots (Fig. 1, Fig. 2). Within the 3 y after the beginning of the experiment (during 1992 to 1994), there seemed to be high recruitment in Ritchie Bay, with the highest occurring in Plot 1, and recruitment was higher in Plots 2 and 4 than in Plot 3 (see Figs. 1, 2). Within the 4 to 8 y after the beginning of the experiment (during 1995–1998), recruitment appeared to be low relative to the period of 1992–1994, with recruitment almost identical among the four plots (see Figs. 1, 2).

During the 10 y before the experiment and in the 1st experimental year of 1992 at Marina Island, recruitment was, in general, the highest in Plot 3 and the lowest in Plot 1 (Fig. 3, Fig. 4). Within the 3 y after the beginning of the experiment (during 1993–1995), recruitment in Marina Island appeared to be strong, but still the highest in Plot 3 and the lowest in Plot 1 (see Figs. 3, 4). Within the 4 to 8 y after the beginning of the experiment (during 1996–1999), recruitment seemed to have generally decreased somewhat from the relatively strong recruitment during 1993 to 1995, but the pattern of recruitment did not seem to have changed. Plot 3 still had the highest recruitment, and recruitment was higher in Plot 2 than Plot 1 (see Figs. 3, 4).

Geoduck recruitment rates were approximately 13 or 14 times higher in the experimental plots of Ritchie Bay than those of Marina Island. The mean densities of 1-y-old geoduck, over all years and plots, were 0.22 per m² in Ritchie Bay and 0.016 per m² for Marina Island.

DISCUSSION

Natural mortality rate (M) is a crucial biologic parameter for understanding geoduck population dynamics. Bradbury and Tagart (2000) found that their yield modeling was most sensitive to the estimates of geoduck natural mortality, and recommended that greater effort be spent on making more reliable estimates of M . We

TABLE 1.
Natural mortality rates (mean \pm standard error) estimated using Monte Carlo simulations (MC) and Bayesian method.

Area	Method	Natural Mortality Rates in Experimental Plots				
		1	2	3	4	Overall
Ritchie Bay	MC	0.084 \pm 0.012	0.027 \pm 0.0055	0.053 \pm 0.018	0.064 \pm 0.0080	0.039 \pm 0.0046
Ritchie Bay	Bayesian ¹	0.065 \pm 0.0066	0.019 \pm 0.0028	0.022 \pm 0.014	0.061 \pm 0.0054	0.036 \pm 0.0024
Ritchie Bay	Bayesian ²	0.064 \pm 0.0089	0.019 \pm 0.0043	0.022 \pm 0.017	0.061 \pm 0.0065	0.036 \pm 0.0027
Marina Island	MC	0.060 \pm 0.032	0.00057 \pm 0.042	–0.23 \pm 0.019		

¹ Without bootstrapping the age structure.

² With bootstrapping the age structure.

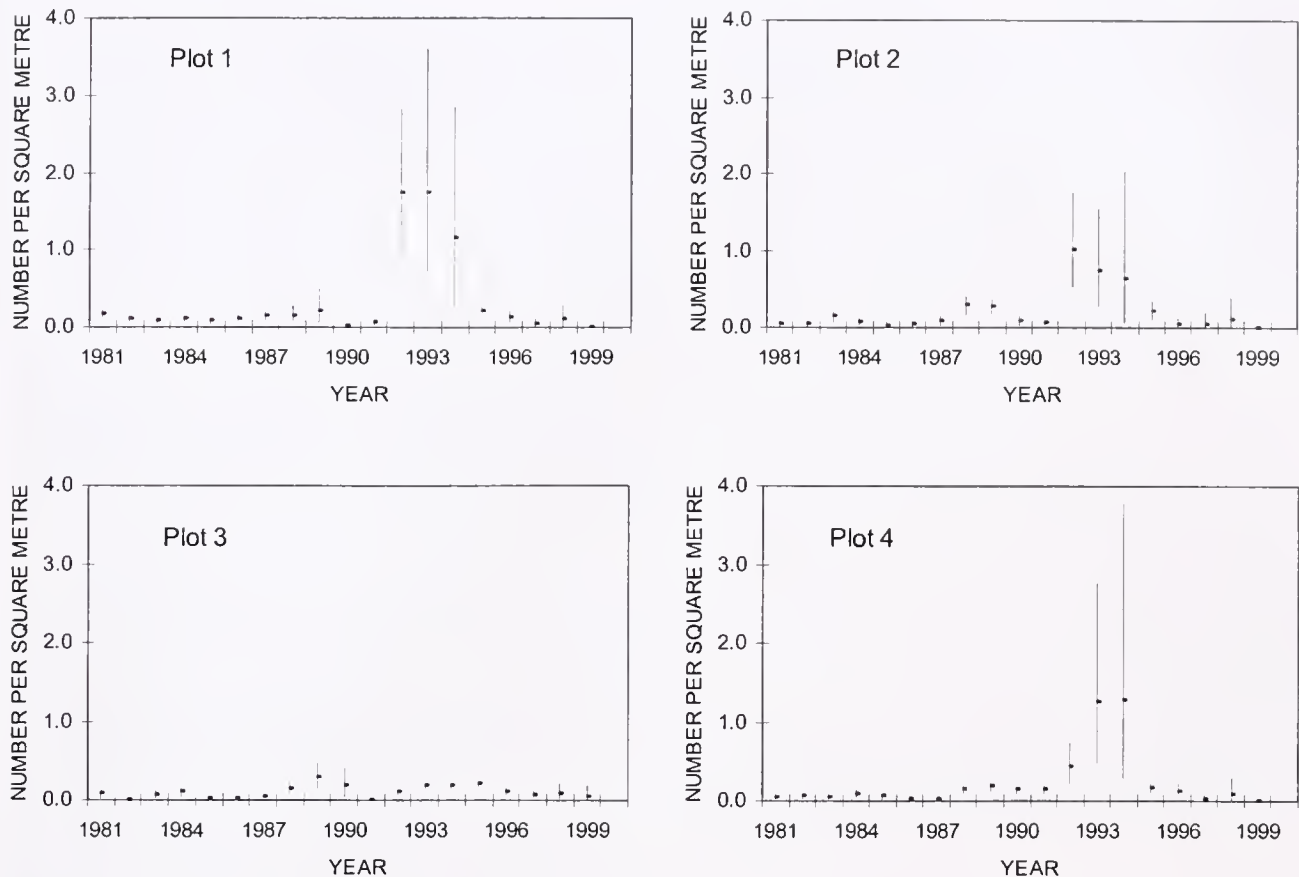


Figure 1. Estimated mean geoduck 1-y-old densities (dots) with 95% confidence intervals (vertical lines) between 1981 and 1999 in each experimental plot at Ritchie Bay.

used a new method to estimate M for geoduck by using age structure modeling, and taking advantage of the time series of density and biosample age frequency data. We included a vulnerability model so that abundance of young geoduck that were not fully vulnerable to divers, could be estimated and included in the comparison of model-derived abundances with survey-derived abundances. Our method was not affected by variation in recruitment, but relied on the accuracy of abundance estimates based on the surveys. In contrast, three other methods have been used in the

estimation of M for geoduck populations. The first and most commonly used method was the catch curve analysis of a range of age frequency distributions selected ad hoc. Values of M for geoduck estimated in this way ranged from 0.01 to 0.04 (Bradbury & Tagart 2000, Breen & Shields 1983, Harbo et al. 1983, Sloan & Robinson 1984, Noakes 1992, Noakes & Campbell 1992). This method is based on the assumption that recruitment is constant over the age range being analyzed, and a shift in recruitment would bias the estimates of M (Ricker 1975, Bradbury & Tagart 2000). There is evidence that geoduck recruitment was low in recent years relative to those in the 1930s (Orensanz et al. 2000). To overcome the impact of temporal variations in recruitment on M estimation, Orensanz et al. (2000) used a second method to analyze age frequency distribution of dead geoduck shells found within the sediments. They estimated M to be 0.036, which was within the reported range of M for geoduck. The third method of estimating M was to trace the fate of individuals *in situ* within a time frame. Bradbury et al. (2000) estimated M to be 0.016 based on the dead proportion of geoduck marked by thin plastic stakes next to the siphons in an unfished site a year earlier. This latter estimate of M was in the low part of the reported range.

The method of this study estimated an overall M for geoduck at Ritchie Bay of 0.036 to 0.039, which was in the upper part of the range reported by previous studies using the first two methods (see earlier). The catch curve analysis of previous studies relied mainly on relatively old geoduck, as few small geoduck were caught and the low cut-off age was usually set to be the age fully vulnerable

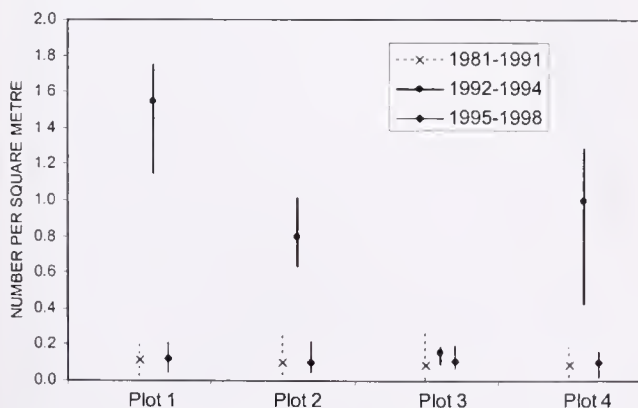


Figure 2. Average (dots) and range (vertical lines) of mean geoduck 1-y-old densities in three different periods in each experimental plot at Ritchie Bay.

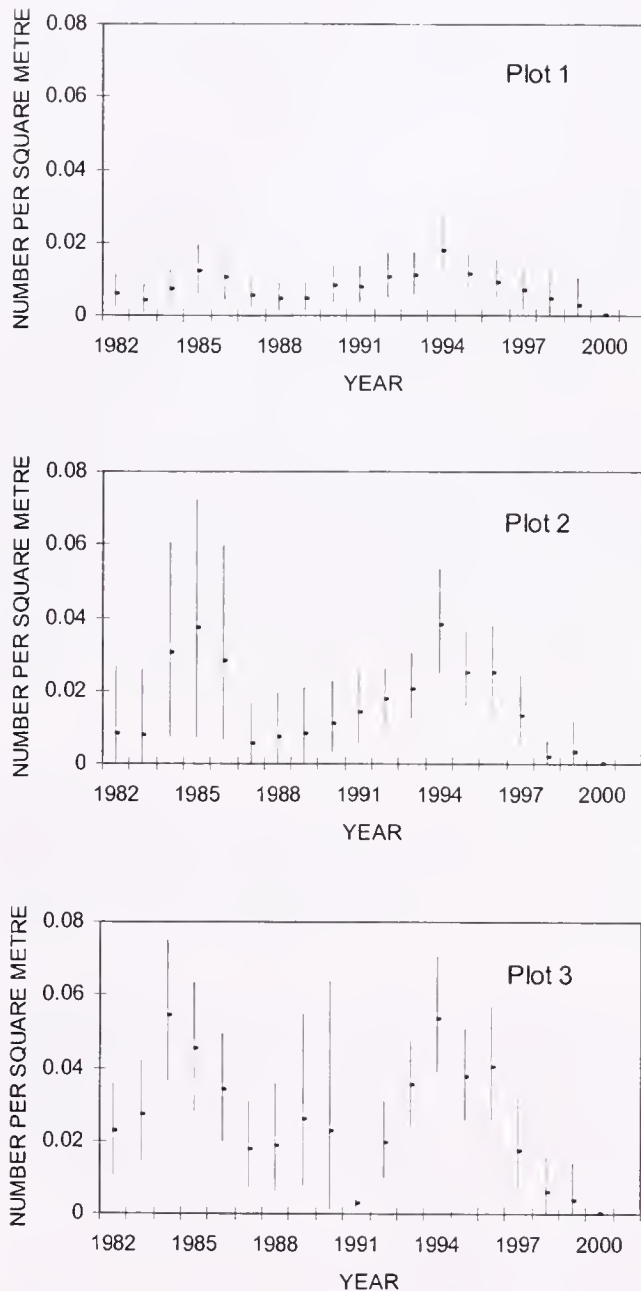


Figure 3. Estimated mean geoduck 1-y-old densities (dots) with 95% confidence intervals (vertical lines) between 1982 and 2000 in each experimental plot at Marina Island.

to fishing. The method of tracing the fate of tagged individuals used geoduck $>3-4$ y (Bradbury et al. 2000). If young geoduck suffer from a higher mortality rate, the previous methods would tend to result in estimates of M that would be somewhat lower than our method, which accounts for young geoduck.

We assessed the variations in estimated M using Monte Carlo Simulations and the Bayesian method. Ideally, ages of bio-samples should be treated as parameters and sequentially sampled in the application of the Gibbs sampler to produce a new set of age structure in each circle of random samplings. However, we did not know the standard errors for age determinations for individual samples. For an approximation, we used bootstrapping to regenerate new sets of age structures. The estimated mean value of M for

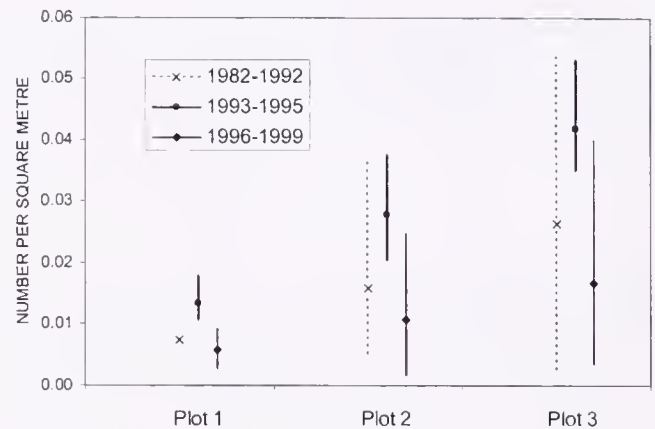


Figure 4. Average (dot) and range (vertical line) of mean geoduck 1-y-old densities in three different periods in each experimental plot at Marina Island.

each plot at Ritchie Bay was almost identical using both techniques, whether bootstrapping was applied or not, although the variances were, expectedly, larger with than without applying the bootstrap method.

Geoduck natural mortality was difficult to estimate from the Marina Island experimental plots using the available time series data. The estimated mean density of geoduck population in Plot 3 increased from 0.03 m^{-2} in 1996 to 0.182 m^{-2} in 2001 based on the surveys (see Fig. 1 of Campbell et al. 2004). The average annual increase in density is 0.0370 m^{-2} , whereas densities of age 7-y geoduck in Plot 3 were estimated to be 0.028 in 1992 and 0.03 in 2001 (See Table 4 of Campbell et al. 2004). The apparent increase in population size in this plot could not be fully contributed by recruitment. Thus, M for Plot 3 could not be meaningfully estimated using the age-structure model. Similarly for Plot 2 at Marina Island, in approximately 50% of the simulations, increases in population size were higher than increases in recruitment. Consequently, the model estimated negative values of M to account for the increases in population size.

To have an effective fishing strategy, one not only needs to know M , but also require an understanding of the impact fishing and population size or density on subsequent recruitment. Bureau et al. (2002) reported that recruitment in recent years appeared to be strong in a number of beds with relatively long harvest histories, suggesting that harvest did not have a negative effect on recruitment. Goodwin and Shaul (1984) found that harvested sites had fewer recruits than the unharvested controls in Port Gamble and Treble Point in Puget Sound, suggesting possible adverse impact of fishing on subsequent recruitment. However, their experiments could not conclusively demonstrate an adverse effect, because the differences were not significant due to the low number of recruits. Unlike our experiments lasting 9 y, the experiment by Goodwin and Shaul (1984) lasted for about 20 mo, which allowed only one geoduck setting season to be observed and the young-of-the-year to grow to the size large enough to be retained by the venturi sampler. Thus, their study could only indicate the possible short-term adverse impacts of fishing on recruitment.

One of the obvious reasons for possible adverse impact of fishing on recruitment was the disturbance of sediment caused by digging into the sediment beside the siphons of geoduck during the fishing (Orensanz et al. 2000). Fishing activity appeared to be highly disruptive of the substrate (Breen & Shields 1983), which

could affect geoduck larval settlement and increase the mortality of newly settled juveniles. We examined and compared geoduck 1-y-old densities that were used as a proxy for relative larval settlement and an index for recruitment of geoduck to the fishery 5 or 6 y later at age 6 or 7 y.

Our study on the impact of harvesting provided new information on recruitment. Recruitment of 1-y-old geoduck during the years before the experiment in Ritchie Bay appeared to be similar among the four experimental plots, suggesting that some physical factors, such as hydrodynamics, or biologic factors, such as predation, played a similar role in larval settlement and or juvenile survival between the four experimental plots. Within the first 3 y (1992–1994) after the beginning of the experiment at Ritchie Bay recruitment appeared to be high. After 62% harvested at the beginning of the experiment in 1991, Plot 1 had a higher recruitment than the two unharvested plots 2 and 4 during 1992 to 1994. The high recruitment in Plots 1 and 2 during 1992 was likely, to some extent, due to artificial seeding of hatchery reared juveniles in 1991 (Campbell et al. 2004). In contrast, Plot 3 had the lowest recruitment during 1992 to 1994, whereas high exploitation occurred during 1991 to 1993 with almost all large geoduck being removed, suggesting that severe harvest might have negatively affected recruitment in the short term (≤ 3 y). Possible short-term negative impacts have also been reported by Goodwin and Shaul (1984). Recruitment rates appeared to be similar again among the four plots 4 y after the beginning of the experiment (i.e., after 1994), offering no evidence that heavy harvesting either positively or negatively affected recruitment in Ritchie Bay in the long-term (> 3 y).

In contrast to Ritchie Bay, Marina Island geoduck recruitment was the highest in Plot 3 and the lowest in Plot 1 during the 10 y before the experiment and the 1st experimental year, suggesting that hydrodynamics was likely affecting larval settlement differently between the three experimental plots. Within the first 3 y (1993–1995) after the beginning of the experiment, the general

differences in recruitment rates among the three plots remained the same as before. The lowest recruitment occurred in the unharvested Plot 1. Despite a heavy 3-y harvest, with almost all geoduck removed during 1992–1994, Plot 3 had better recruitment than either moderately harvested Plot 2 or non-harvested Plot 1 during 1993 to 1995. This pattern of recruitment was also observed 4 y after the beginning of the experiment. The impact of harvesting, if there was any, was probably largely masked by physical factors such as the local hydrodynamics differentially providing different larval settlement supply. Other factors, such as predation also may have affected post settlement survival. We concluded that at Marina Island low geoduck densities throughout the 9-y experiment could provide little evidence of either positive or negative impact of fishing on recruitment.

The age-structure model was a useful tool for estimating historical recruitment patterns and M in individual geoduck beds. The value of M estimated by the age-structure model was likely to be more accurate than estimated by the method of catch curve analysis of age frequency distributions, because the age-structured model takes into account the temporal variations in recruitment and young geoduck not fully vulnerable to fishing. The constant recruitment assumption, required of the method of catch curve analysis, is unlikely to be an accurate assumption especially for the long-lived geoduck. Also, an age range has to be chosen for the catch curve analysis, and selection of different age ranges is likely to result in different M values. Use of the age-structure model, however, requires surveys for density and biosamples for age frequencies in the same beds over multiple year intervals, which could be achieved through long term planning for fisheries assessment purposes.

ACKNOWLEDGMENTS

The authors thank the Underwater Harvesters Association for logistic support in data collection, and Lyse Godbout and an anonymous reviewer for comments that improved this paper.

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POPULATION ABUNDANCE ESTIMATES OF THE NEW ZEALAND GEODUCK CLAM, *PANOPEA ZELANDICA*, USING NORTH AMERICAN METHODOLOGY: IS THE TECHNOLOGY TRANSFERABLE?

PAUL EDWARD GRIBBEN,^{1*} JEREMY HELSON² AND RUSSELL MILLAR³

¹School of Environmental and Marine Sciences, and Leigh Marine Laboratory, University of Auckland, Private Bag 92019, Auckland, New Zealand; ²Island Bay Marine Laboratory, School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand; ³Department of Statistics, University of Auckland, Private Bag 92019, Auckland, New Zealand

ABSTRACT This study investigates the applicability of methods used to describe patterns of distribution, and estimates of density and biomass of the Pacific geoduck clam, *Panopea abrupta*, from western North America for populations of the New Zealand geoduck, *Panopea zelandica* in Kennedy Bay, on the Coromandel Peninsula, and in Wellington Harbour. Central to this is the use of line transect surveys and estimations of the detection rate of geoduck (show-factor) using counts of siphon holes. Studies were restricted to less than 17 m water depth. Geoduck in Kennedy Bay were found from 4–8 m water depth, whereas geoduck in Wellington Harbour occurred in several separate beds from 4–16 m. In Wellington Harbour, there was a pattern of increasing numbers with depth up to ~15 m. Numbers seemed to decrease thereafter. Analysis of sediment samples indicated that *P. zelandica* was more prevalent in fine sand to fine silty sand substrates. *P. abrupta* is found in similar habitats. There was no significant difference in the show-factor (the proportion of geoduck detectable by sight or touch vs. the actual number of geoduck present) of geoduck with respect to season (summer and winter), region (Wellington Harbour and Kennedy Bay), or tidal height (low, mid, and high tide). Hence, a mean show-factor of 0.914 was used to adjust density estimates from all surveyed populations. Estimates of the mean population density (\pm SE) of *P. zelandica* were much lower than those reported for *P. abrupta* ranging from 0.058 (\pm 0.01) geoduck/m² in Kennedy Bay to 0.489 (\pm 0.08) geoduck/m² in Shelly Bay, Wellington Harbour. Survey densities, abundance, and biomass estimates were reasonably well determined with coefficients of variation (CVs) generally less than 20%. The results suggest that the methods used to provide population estimates for *P. abrupta* are readily transferable to *P. zelandica*. However, further research needs to be conducted on the diver variability on counts of geoduck, the role that geoduck occurring in water depths >17 m play in the population dynamics of local populations, and the density dependence of fertilization success of *P. zelandica*. Given the low estimates of density in this study, fisheries managers will have to carefully consider the feasibility of sustainably harvesting this species.

KEY WORDS: abundance, biomass, density, detectability, habitat, hiatellidae, mollusca, *Panopea zelandica*, show-factor

INTRODUCTION

Geoduck are one of the largest and longest-lived clams in the world (Beattie & Blake 1999). The Pacific geoduck clam, *Panopea abrupta* (Conrad 1899) (= *generosa*), from western North America can reach ages in excess 100 years (Goodwin & Shaul 1984, Sloan & Robinson 1984) and individuals as large as 3.25 kg have been recorded (Goodwin & Pease 1991). The only other species for which detailed biological information is available is the New Zealand geoduck, *Panopea zelandica* (Quoy & Gaimard 1835). Although not as large as *P. abrupta*, individual *P. zelandica* can reach 600 g and 80 years of age (Gribben & Creese, in press).

Commercial harvesting of *P. abrupta* forms the most important clam fishery on the Pacific Coast of North America. Large numbers are found in Puget Sound, Washington, and British Columbia, where subtidal stocks have been exploited since the 1970s. The combined Washington and British Columbian fisheries are worth US\$35 million annually (Harbo 1998, Hoffman et al. 2000). A small fishery established in 1988 for *P. zelandica* was closed in the early 1990s pending its introduction into the quota management system (QMS) (Breen 1994). Recently, there has been renewed interest in establishing commercial fisheries and aquaculture industries because of the similarity of *P. zelandica* to *P. abrupta*.

Fisheries for *P. abrupta* are based on detailed habitat descriptions and established methods for providing accurate density and biomass estimates (Hand & Dovey 1999, 2000). Geoduck density is estimated by counting the number of siphon holes visible at the

sediment surface. However, this can lead to an underestimation of the number of geoduck present as not all geoduck show at the same time (Goodwin 1977, Hand & Dovey 1999). Central to obtaining accurate density estimates is the use of show-factors. The show-factor is the proportion of geoduck that is visible or can be felt below the sediment surface versus the total number present in control plots. Any estimates of geoduck density obtained using diver transects are then adjusted by the appropriate show-factor. These have been shown to vary seasonally (Goodwin 1977), tidally (Hand & Dovey 1999) and with storms (Campbell et al. 1996a).

Most of the current literature available for *P. zelandica* concerns estimates of age, growth and mortality (e.g., Breen et al. 1991, Gribben & Creese, in press), as well as descriptions of larval (Gribben & Hay 2003) and sexual development (Gribben & Creese 2003, Gribben et al. 2004). *Panopea zelandica* is known to occur throughout New Zealand's three main islands in subtidal sand and mud habitats (Morton & Miller 1973, Powell 1979). However, the distribution and general habitat preference of local populations remains poorly described. Moreover, the applicability of methods used to provide reliable density and biomass estimates for *P. abrupta* is also yet to be investigated. Understanding the environmental requirements and providing realistic estimates of the density for local populations will be the first step in developing sustainable management policies and for assessing the suitability of *P. zelandica* for culture (Malouf & Bricelj 1989, Murawski & Serchuk 1989).

This study describes the distribution and general habitat preference (i.e., sediment type and water depth) of populations occurring in Kennedy Bay, on the Coromandel Peninsula, and Well-

*Corresponding author. E-mail: p.gribben@unsw.edu.au

ton Harbour, and also investigates the show-factors of *P. zelandica* occurring in these same populations. It also determines whether established methods of providing density estimates for *P. abrupta* (i.e., counts of siphon holes using diver transect surveys) are readily transferable to *P. zelandica*, and whether they can be used reliably to provide further population abundance/biomass estimates.

METHODS

The harvestable biomass of *P. abrupta* is considered to be only that part of the population that occurs in waters shallower than 17 m below chart datum. This is due to the risks posed to divers spending considerable lengths of time underwater at depths greater than this. Thus all research in this study was conducted in waters no deeper than 17 m.

Relationship Between Gross Environmental Characteristics and Distribution

Transect surveys were used to determine the distribution and sediment characteristics of local *P. zelandica* populations occurring in Kennedy Bay, on the Coromandel Peninsula, and Shelly Bay, in Wellington Harbour (Fig. 1A to C). Similar methodologies were used for describing the distribution of local *P. abrupta* populations in North America (Hand & Dovey 1999, 2000).

Kennedy Bay is a large circular, sheltered bay 1.5 km in diameter, which is shallow and gently sloping with a maximum depth of 11 m at the bay entrance. Kennedy Bay is generally only

exposed to winds from the north-northeast. Shelly Bay, a small sheltered embayment located within Wellington Harbour, is more sheltered, although steeper in slope, than Kennedy Bay and can reach depths of 20 m within 60 m of the sea shore.

In Kennedy Bay, five transects 1400 m long and 250 m apart (designed to encompass the majority of the subtidal area of the bay) were sampled from Sept 29 to Oct 1, 1999. Eight stations 200 m apart (as determined by DGPS prior to the beginning of the survey) were sampled along each transect. The number of geoduck in $16 \times 1\text{-m}^2$ quadrats was determined at each sample station. The large number of quadrats searched reflected the low density of geoduck found in this bay observed from previous exploratory studies (Gribben unpubl. data). Sediment samples were collected at all stations using a hand held corer. Approximately 600 g of sediment was extracted for analysis at each station using a corer 5 cm in diameter pushed a depth of 100 mm into the sediment. In Shelly Bay, five transects 50 m apart (determined by running a tape measure parallel to the shoreline) were sampled from Oct 26 to 28, 1999. Transects started at the 5 m depth contour, except for the first transect which began at the 2.5 m depth contour. Sediment samples were collected every 15 m along a tape measure run perpendicular to the shore. Transects ranged in length from 75–120 m, with $4 \times 1\text{-m}^2$ quadrats searched for geoduck at each station. Lower numbers of quadrats were used in this survey because geoduck were much more abundant in this area (Gribben unpubl. data). Each transect ended between the 13–16-m depth contours depending on when the sediment became too silty, making it difficult to effectively search quadrats for geoduck. All sediment samples collected from Kennedy Bay and Shelly Bay were put in labeled plastic bags and transported back to the Leigh Marine Laboratory where they were dried in an oven at 60 °C for 3 days. Samples were sieved using a vibrating shaker through a graded series of seven sieves: mesh sizes <125 μm , 125 μm , 250 μm , 500 μm , 1 mm, 2 mm, and 4 mm (Wentworth grade classification) (Ingham 1971). Median phi size was calculated for each sample as per the methods outlined in Buchanan (1984). The results are displayed as bubble plots of geoduck density versus water depth and median phi size for both populations (Sigma Plot, SPSS 2000).

Investigation of Show-Factors

The show-factor of *P. zelandica* was investigated in Shelly Bay and Mahunga Bay, Wellington Harbour, and in Kennedy Bay (Fig. 1). The general methods are as follows. Depending on the experiment (see below), a number of semipermanent quadrats were placed in each population in areas of known high density (as determined from the survey above) to increase precision. Each quadrat was visited for 5 consecutive days. The first time a quadrat was visited the geoduck present (those whose siphons could be felt or seen) were recorded and staked. On subsequent visits any geoduck not previously recorded were also counted and staked. Estimation of show-factor proportions follows the methods outlined in Hand and Dovey (1999). The proportion showing on any day, SP_i , in any given quadrat is calculated as

$$SP_i = X_i / \sum_i T \quad (1)$$

where X_i is the number of geoduck showing on day i and T is the total number of geoduck observed, assuming that no geoduck remained hidden for more than 5 consecutive days (justification for

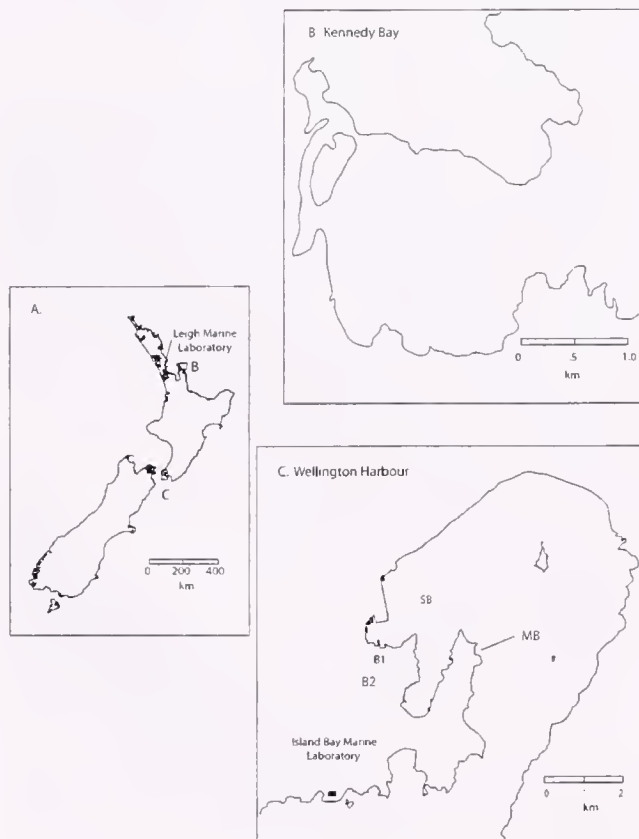


Figure 1. Map of New Zealand (A) indicating the position of study sites in Kennedy Bay (B), and Wellington Harbour (C), including Shelly Bay (SB), Mahunga Bay (MB), Bay 1 (B1) and Bay 2 (B2).

this is given in the discussion). The standard error of the estimate ($se(SP_i)$) is approximated by

$$se(SP_i) = \sqrt{\frac{(SP_i(1 - SP_{it}))}{\sum T}} \quad (2)$$

because SP_i is binomially distributed.

Two studies were conducted in Mahunga Bay and Shelly Bay, in Wellington Harbour. The first study investigated the show-factor of geoduck at different tidal heights (slack low tide and slack high tide), seasons (summer and winter) and sites (Mahunga Bay and Shelly Bay). The winter survey was conducted from June 26 to July 1, 1999, and the summer survey from Jan 29 to Feb 4, 2000. Five replicate 4 m² quadrats per treatment were placed in areas of high density within each population. The second study, conducted from Oct 17 to 21, 2000, investigated whether the show-factors at the two sites differed between slack tide or mid tide: there was no seasonal component to this experiment. However, the quadrats used in the first study were too large to search efficiently because disturbed sediment obscured the search for geoduck before the quadrat was completely searched. Instead, 10 × 1-m² quadrats were used for each treatment combination at each site.

In Kennedy Bay, studies one and two, described above, were combined. The effect of tidal flow (slack low, slack high, and mid tide) and season on show-factors was investigated in summer from Jan 19 to 23, 2001 and in winter from July 23 to 27, 2001. Thirteen replicate 1 m² quadrats were used for each treatment combination. The show-factors were modeled as binomial proportions using logistic regression (PROC Logit, SAS 1988) to investigate whether they were influenced by tidal flow, season or location.

Population Estimates

Estimates of area, density, abundance, and biomass of geoduck populations in Kennedy Bay, Shelly Bay, and Mahunga Bay were provided once the initial distribution surveys had been conducted. Surveys of population estimates in Wellington Harbour were extended to include 2 bays immediately to the south of Shelly Bay, hereafter referred to as Bay 1 and Bay 2 (Fig. 1A to C).

In the Wellington Harbour sites, transects (20 m apart) of a known length and width (1 m) were placed in each population to provide area and density estimates. Although the transects were spaced at regular intervals, the first transect was placed randomly at the either northern or southern perimeter of the bay. This effectively renders all transects random. All transects began at 6 m below chart datum and ran to 15 m. The length of each transect was recorded with a tape measure. All geoduck encountered along the tape measure within the bounds of a 1-m stick centered on tape measure were recorded. A total of 11 transects were run in Mahunga Bay, 10 transects in Shelly Bay, 6 transects in Bay 1, and 10 transects in Bay 2. In Kennedy Bay, a total of 14 transects, 600 m long and ~60 m apart were run through the population as defined in the initial survey. The starting point for each transect was determined by DGPS prior to the beginning of the survey. Again, although the transects were regularly spaced, the accuracy with which this could be done effectively renders the transects random (Hand et al. 1998a).

Area Estimates

Estimates of area follow the methods outlined in Hand and Dovey (1999). The surveyable area in each bay was defined to be

the sum of the area of all the possible transects. Because transect length was only known for those sampled, the length of transects not sampled was assumed to be equal to the length of the nearest sampled transect (Hand & Dovey 1999). Thus, the surveyable area A (m²) is

$$A_j = \sum_{i=1}^n L_i(W1_i + W2_i) \quad (3)$$

where n is the number of transects sampled in bed j , L_i is the length (m) of the i^{th} transect, and $W1_i$ and $W2_i$ are the distances (m) on either side of the transect i , equidistant to its adjacent transect. That is, as transects are 20 m apart in Wellington Harbour and 60 m in Kennedy Bay, both $W1_i$ and $W2_i$ are 10 m in Wellington Harbour and 30 m in Kennedy Bay.

Density Estimates

A common show-factor was assumed for all transects within a bed. Estimates of mean geoduck densities were obtained by estimating the mean density of observed geoduck over a bed, and then adjusting for the show-factor. The mean density of geoduck observed in a bed, o_j , was estimated using the ratio estimator method because it reduces the variance of the estimate arising due to unequal transect lengths by weighting the transect counts according to the transect length (Hand & Dovey 1999). This gives

$$o_j = \frac{\sum_{i=1}^n b_i}{\sum_{i=1}^n a_i} \quad (4)$$

where b_i is number of geoduck found along a transect and a_i is the total area of transect i in square meters. The standard error of o_j is given by

$$se(o_j) = \sqrt{\frac{\sum_{i=1}^n (b_i - o_j a_i)^2}{n(n-1)\bar{a}^2}} \quad (5)$$

where

$$\bar{a} = \frac{1}{n} \sum_{i=1}^n a_i$$

is the average area of the transects (Campbell et al. 1998a, Hand & Dovey 1999).

Adjusting for the show-factor gives the estimate of mean adjusted geoduck density (d_j) is

$$d_j = o_j / SP \quad (6)$$

where SP is the estimated show-factor (Campbell et al. 1998a, Hand & Dovey 1999). The estimation variability of SP needs to be considered when calculating the standard error of d_j . This is done using the formula

$$se(d_j) = \quad (7)$$

after Thompson (1992).

Population Density and Biomass Estimates

Estimates of total numbers (± 1 SE) of geoduck in each bed were calculated from the estimates of mean density (m⁻²) and total surveyed area of each population. Total biomass (± 1 SE) of geo-

duck in each population was determined from estimates of total abundance and mean whole wet weight (± 1 SE).

Estimates of mean whole wet weight (g) (± 1 SE) of geoduck in Kennedy Bay and Wellington Harbour were determined by haphazardly collecting geoduck by SCUBA Kennedy Bay ($n = 153$) during January 1999 and from Shelly Bay ($n = 113$) during June 1999. Whole wet weight was measured to the nearest 0.1 g, using a Mettler electronic balance for all individuals. All clams were processed within 15 minutes of capture. The mean whole wet weight from Shelly Bay was assumed to be representative of all bays in Wellington Harbour. The standard error of the product of mean weight (W) and total abundance (N) was estimated by the equation

$$S.E.(NW) = \sqrt{W^2 s_N^2 + N^2 s_W^2} \quad (8)$$

as given in Topping (1962).

RESULTS

Environmental Characteristics

The mean density of geoduck ranged from 0 to 3.5 geoduck/m² at stations sampled in Shelly Bay, and from 0 to 0.44 geoduck/m² for stations sampled in Kennedy Bay (Fig. 2). Geoduck in Shelly Bay were found between 4–16 m below sea level, although few geoduck were found in water shallower than 6 m and deeper than 15 m. It is noted that few stations deeper than 15 m were sampled. Geoduck were not evenly distributed throughout their depth range and appeared to be clumped into 2 main areas, one between 6–8 m below sea level and another between 10–15 m. The low number of geoduck from 8–10 m corresponds with an area that has larger amounts of shell on the surface of the sediment, which runs through this bay at these depths (Gribben pers. obs.). The distribution of geoduck in Kennedy Bay was mostly confined to an area approximately 1000 \times 600 m (Fig. 3). The depth range (~3.5 to 8 m) of geoduck in Kennedy Bay was more restricted than that in Shelly Bay (Fig. 2).

Median phi size in Shelly Bay was variable, ranging from minus 3 to 2.8 (large stones to sand-silt sediments). The narrower

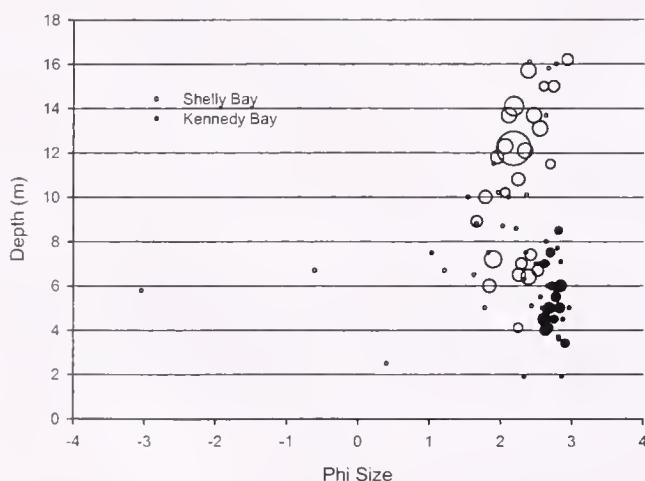


Figure 2. Plot of geoduck density (m⁻²) in relation to Phi size and water depth in Kennedy Bay (black circles) and Shelly Bay (clear circles). Circles are mean density of geoduck sampled at each station. Smallest circles = no geoduck, median circles = 1.75 m⁻² and largest circles = 3.5 m⁻².

range of phi sizes in Kennedy Bay, 1.0 to 2.9 (sand to fine sand sediments) reflected the more homogenous nature of sediments in this area (Fig. 2). Geoduck in Shelly Bay were only found in sediments with a median phi size between 1.8 and 2.5 (fine sand to silty fine sand sediments) (Fig. 2). No geoduck were found in sediments coarser than <1.8. Geoduck in Kennedy Bay were not found in sediments coarser than 2.5, although few stations were of that type. This study ignored any patterns of abundance that may have existed for geoduck in Shelly Bay occurring in waters deeper than 16 m.

Show-factors

The mean proportions (\pm SE) of geoduck showing in Wellington Harbour and Kennedy Bay are summarized in Figures 4 and 5 respectively. In Wellington Harbour, few new geoduck were observed after day 3 in experiment 1 (Fig. 4A,B), whereas no new geoduck were found after day 2 in experiment 2 (Fig. 4C). The change of experimental design seems justified because the proportions of geoduck found on the first visit were significantly higher ($P < 0.005$) in experiment 2 compared with experiment 1. No new geoduck were found in Kennedy Bay after day 2 (Fig. 5).

The absence of any significant differences in show-factors within either region allowed investigation of possible differences that may have existed between Kennedy Bay and Wellington Harbour. The Kennedy Bay pooled data set was tested against a Wellington data set containing only the pooled data (including both Mahunga Bay and Shelly Bay) from experiment 2, as it followed the same methodology as that in Kennedy Bay. There was no significant difference in the show-factor between the two data sets ($P = 0.99$). As a consequence, a show-factor of 0.914 (± 0.019), estimated from the combined Wellington Harbour and Kennedy Bay data sets, was used to adjust total geoduck found and density, abundance, and biomass estimates from both regions (see below). The standard error of the total number of geoduck encountered for each transect was provided by equation 7 (see earlier).

Population Estimates

Mean transect density of geoduck in Kennedy Bay ranged from 0.01 to 0.13 geoduck/m², and in Wellington Harbour ranged from 0.04 to 1.14 geoduck/m² (Table 1). Geoduck were found on all transects sampled in all populations except for transects 5 to 10 in Bay 2. Thus, estimates of area, abundance, density, and biomass for this bay were determined for the first four transects only. Also, because of the very low number of geoduck found along transects 12 to 14 in Kennedy Bay, they were considered to be outside the bounds of the main population and were excluded from further analyses.

The population of geoduck in Kennedy Bay occupied the largest area (39.6 ha) of all of the five bays sampled (Table 2). Mean geoduck density was highest in Shelly Bay and lowest in Mahunga Bay. Kennedy Bay had the highest estimated abundance and biomass of geoduck and B1 the lowest, mainly due to the size of the area surveyed. Although Kennedy Bay was over an order of magnitude larger in terms of area than Shelly Bay, the overall biomass was only approximately 60% larger. This was a result of the lower mean density and smaller estimated weight found in Kennedy Bay.

DISCUSSION

There is considerable interest in developing fisheries and aquaculture industries for geoduck species throughout Asia and the

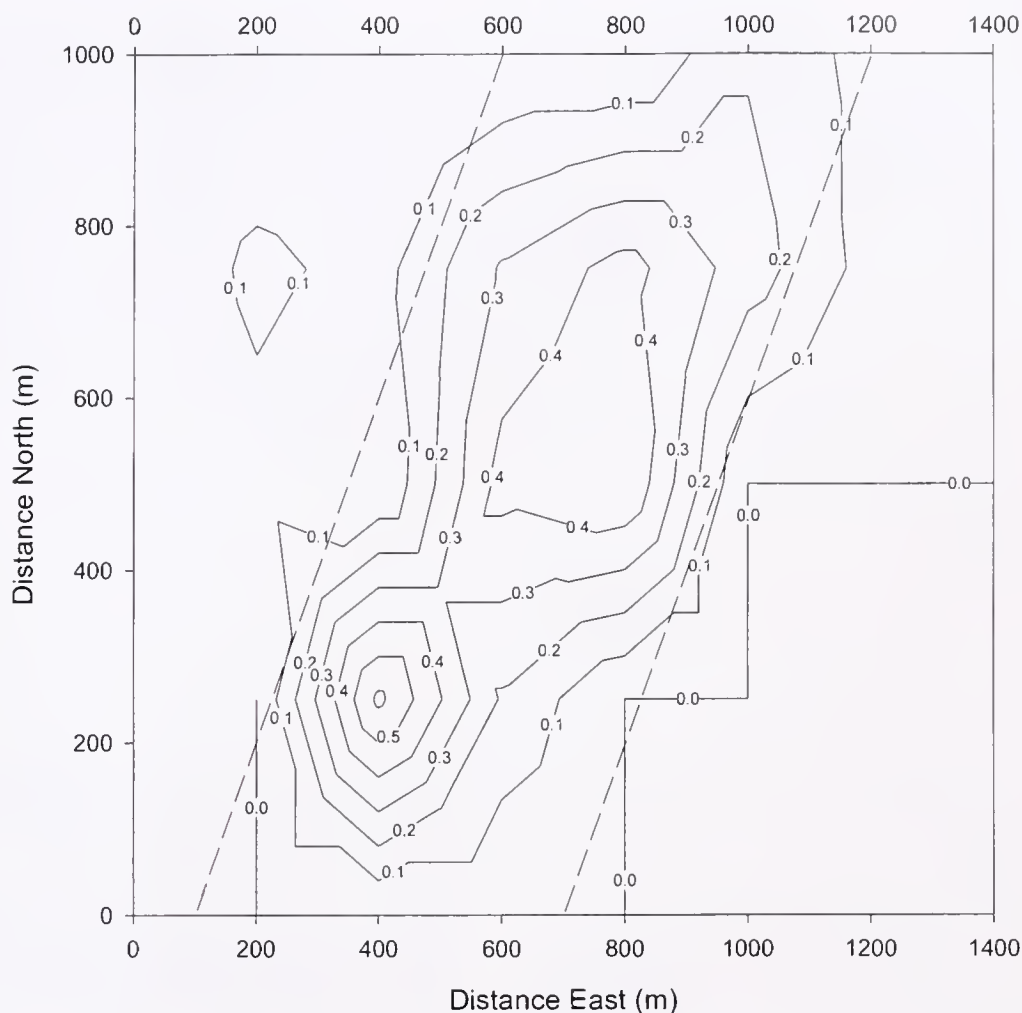


Figure 3. Contour plot of the distribution and density (not adjusted for show-factors) of geoduck (number m⁻²) in Kennedy Bay. Dashed lines indicate the main area of the bed used in subsequent analyses.

Pacific. Therefore, studies investigating the transferability of existing methodologies for determining population size and structure for *P. abrupta* from North America are both timely and necessary. This is the first study to describe the distribution, density and biomass of local populations of the New Zealand geoduck, *Panopea zelandica*, although this study was restricted to the harvestable population at <17 m water depth.

Morton and Miller (1973) and Powell (1979) described *P. zelandica* as an ocean beach mollusk found in sand and mud habitats throughout New Zealand. This study indicated that *P. zelandica* habitat range was more diverse than previously described. All the populations of *P. zelandica* investigated in this study occurred in sheltered bays indicative of more benign conditions, a habitat similar to that described for *P. abrupta* (Goodwin & Pease 1989). Other populations not investigated in this study occur at similar depths and environments (e.g., Golden Bay, Nelson, Gribben unpubl. data).

Studies have shown that the density of *P. abrupta* in Puget Sound, Washington, was higher in sand or mud-sand habitats than in mud, pea-gravel, or gravel substrates (Goodwin & Pease 1989). Other research has shown that density was positively correlated with water depth from 0–25 m, decreasing thereafter (Goodwin & Pease 1989, 1991; Campbell et al. 1996b). Similar results were

obtained for *P. zelandica* in this study. Although, our research only considered that part of the population that was harvestable, searches for geoduck at in silty sediments at depths greater >17 m indicated they were scarce (Gribben unpubl. data). However, the level to which geoduck found at greater depths contribute to the population dynamics of local populations is unknown and requires further investigation (Hand & Dovey 1999). Potentially, these harvest refugia could be an important source of larval recruits to the shallower fished stocks.

Although not quantified, observations suggested that geoduck in all bays appeared to be more abundant in areas that were covered in a film of benthic algae during spring and summer (Gribben unpubl. data). In Kennedy Bay, this region was in 5–6 m water depth and in the Wellington Harbour populations this occurred around 10–12 m. This may explain why geoduck in Kennedy Bay were mainly restricted to depths of 4–6 m when most of the bay appeared to have relatively homogenous sediments. The biofilms may provide a settlement cue for competent larvae as has been shown other marine bivalves (reviewed in Wicczorek & Todd 1998). Populations of *P. zelandica* spawn from spring to late summer so larvae would be in the water column and competent to settle during this period (Gribben et al. 2004). In a laboratory study, *P. zelandica* were shown to have a larval development

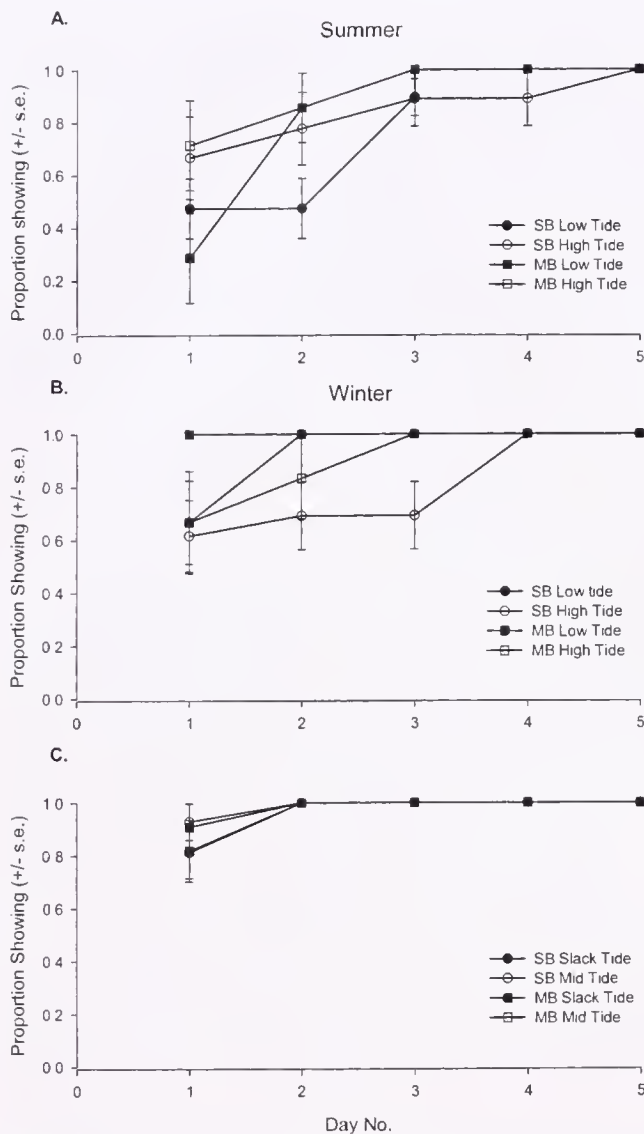


Figure 4. Cumulative proportion (\pm SE) of geoduck showing in show-factor plots in Shelly Bay (SB) and Mahunga Bay (MB), Wellington, during experiment one conducted during summer (A) from 29/01/2000 to 4/02/2000, winter (B) from 26/06/1999 to 1/07/1999, and experiment two (C) from 17-21/10/2000. Means are dots and vertical lines are \pm 1SE.

period of 16 days when maintained in water of 17 °C (Gribben & Hay 2003). Further to this, in studies on the feeding ontogeny of newly settled geoduck, *P. abrupta* were found to have incomplete siphons and used their palps to pass sediment surface material into their mouths (King 1986). Thus benthic algal mats may also provide a potential food source newly settled geoduck.

The counting of geoduck siphons is preferable to removing samples from the substrate because of the depth to which they bury, as removing them from the sediment and replacing them can lead to high levels of mortality (Gribben & Creese, in press). However, individual geoduck siphons are not consistently visible throughout the day, season or year (Goodwin 1973, 1977; Fyfe 1984; Campbell et al. 1998b). Consequently, area and time specific show-factors are required to correct the density counts in British Columbia and Washington State. Mean show-factors for *P. ze-*

landica were consistently above 90% regardless of the season, site, and time of tide, suggesting that this value may be generally applicable. However, our calculation of show-factors is based on the assumption that all geoduck do not remain hidden for more than 5 days. We believe that this is a valid assumption for the following reasons. Many studies on *P. abrupta* consistently indicated high show-factors (>90%) during summer months (Fyfe 1984, Hand & Dovey 1999, 2000). On a smaller scale, Hand and Dovey (1999, 2000) visited show-factor plots of *P. abrupta* of known abundances for 8–9 consecutive days. Show-factors were high on any given day (71% to 100%), indicating that nearly all geoduck were showing each day of the survey. In our study, once found a geoduck was visible on all subsequent days. This indicates that only reason that geoduck were not located initially was due to poor visibility or they were inadvertently missed.

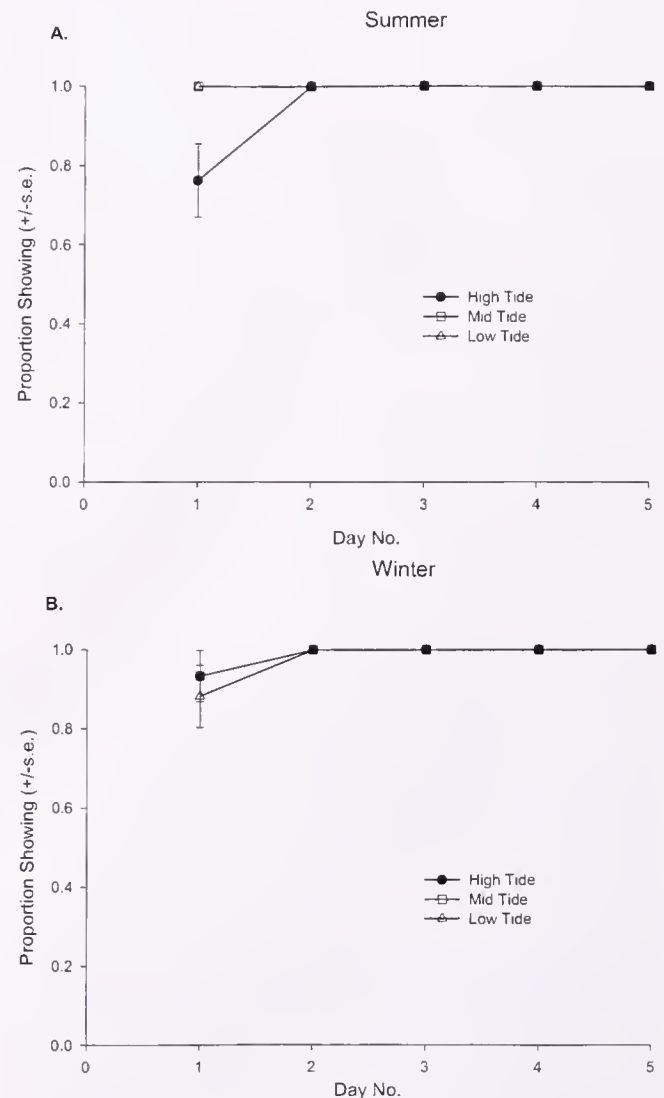


Figure 5. Cumulative proportion (\pm SE) of geoduck showing in show-factor plots in Kennedy Bay during summer (A) and winter (B) at high, mid, and low tides sampled from 26/6/1999 to 1/7/1999. Means are dots and vertical lines are \pm 1SE.

TABLE 1.

Transect no. (T), length of transect (m) (L) and number of geoduck (no.) along each transect sampled in each location.

T	Population									
	Kennedy Bay		Shelley Bay		Mahunga Bay		Bay 1		Bay 2	
	L	No.	L	No.	L	No.	L	No.	L	No.
1	600	12	100	46	170	20	45	2	105	54
2	600	44	100	32	145	16	40	6	70	38
3	600	78	100	24	60	12	40	6	60	26
4	600	36	115	34	100	10	35	18	60	4
5	600	34	100	46	100	18	35	40	60	0
6	600	36	100	46	55	8	40	12	60	0
7	600	28	80	52	85	14			80	0
8	600	16	85	86	100	4			75	0
9	600	22	80	36	105	12			85	0
10	600	28	75	16	65	8			95	0
11	600	16			100	14				
12	600	4								
13	600	4								
14	600	2								

Although several studies on *P. abrupta* have shown that show-factors are depressed during winter (Goodwin 1973, 1977; Fyfe 1984), winter is a period of active gametogenic development in populations of *P. zelandica* (Gribben et al. 2004). If *P. zelandica* were remaining inactive for extended periods of time, then it seems unlikely they could meet all metabolic needs, including gamete production during winter. Fyfe (1984), in a study of geoduck in Ritchie Bay, British Columbia, found that *P. abrupta* may lay "dormant" for periods of up to 2 months, possibly in response to low winter temperatures and food availability (Goodwin 1973, 1977; Fyfe 1984). However, the plots were only visited once a month during the winter period. Fyfe (1984) assumed that previously tagged geoduck not visible at the next sampling period had remained inactive for at least that period. This may certainly not have been the case. In fact, gametogenesis in *P. abrupta* from both Washington (Andersen 1971, Goodwin 1976, Beattie & Goodwin 1992) and British Columbia (Sloan & Robinson 1984) also progresses rapidly through autumn and winter. Hence, it would also seem unlikely that *P. abrupta* would remain inactive for extended periods.

This study indicated the benefit of using smaller sized quadrats when assessing show factors, as shown by the significant increase in show-factors between experiments 1 and 2 in Wellington Harbour. The smaller quadrats used in experiment 2 were easier and quicker to search. The poor show-factor and large confidence intervals in experiment 1 were due to the large size (2 m × 2 m) of the quadrats and the silty nature of the sediment. The time needed to search the quadrats increased the disturbance of the sediment making geoduck difficult to find. However, the 1-m² quadrats used in subsequent experiments could be further refined into narrow strip transects (approx. 1 m × 10 m), similar to those used in the North American surveys (e.g., Hand & Dovey 1999, 2000) that would allow increased numbers and a larger area to be surveyed.

In Puget Sound, a show-factor of 0.75 is currently used to adjust density estimates on all beds for refishing surveys unless a show plot is established (Bradbury et al. 2000). This is strictly a

management decision assumed to give a conservative estimate of harvestable biomass. The common 0.914 show-factor presented here suggests that this adjustment may be applicable to other populations of *P. zelandica*. However, more research needs to be conducted on the applicability of a general show-factor to other populations, especially those found in cooler waters. All geoduck counts in this study were conducted by a single experienced geoduck diver.

Density and Biomass Estimates

The setting of annual quotas for *P. abrupta* is reliant on reliable estimates of density and area, which vary geographically (Sloan 1985). Every parameter used in calculating virgin density, abundance, and biomass is estimated with varying uncertainty (Hand & Dovey 1999). Survey densities, abundance, and biomass estimates were reasonably well determined with coefficients of variation (CVs) of less than 20% except for the two smallest bays for which the CVs were approximately 30%. This may have been due to the small number of transects surveyed within the bounds of the populations. Increasing sample sizes (i.e., running more transects within the bays) may have helped reduce the CVs.

Estimates of the average densities of geoduck populations in British Columbia have ranged from 0.2 to 5.0 geoduck/m² (Breen & Shields 1983, Hand et al. 1998a, Hand et al. 1998b). Densities as high as 30 m⁻² have been recorded in Griffith Harbour, British Columbia (Hand & Dovey 2000). Average density based on 8589 transects in Puget Sound was 1.7 m⁻² and ranged from 0–22.5 geoduck/m² (Goodwin & Pease 1991). The mean population density estimates provided for *P. zelandica* in this study are lower than those estimated for the *P. abrupta* (Table 2). Although large in area, the low estimated density in Kennedy Bay would appear to make harvesting in this bay unsustainable. In Wellington Harbour, the mean density was much higher although the combined area of geoduck occupied in the regions surveyed was only ~5.5 ha. However, this is likely to be only a portion of the total population in the harbor, as many other bays with similar habitats to those surveyed were not explored. Geoduck will probably not be harvested from this harbor for reasons of pollution. Other larger populations are known to occur in the Marlborough Sounds, and Golden Bay, Nelson. Studies investigating the density, abundance and biomass of geoduck in this area are yet to be conducted.

Fisheries managers will have to be particularly careful in determining whether populations of *P. zelandica* can be harvested sustainably. Harvesting geoduck results in a thinning of the population, with the densest areas the most heavily fished. Evidence suggests that recruitment into North American geoduck populations has been falling for the past 20 years (Orensanz et al. 2000), although some several recruitment events have occurred in British Columbia in the last decade (Bureau et al. 2002). Because *P. zelandica* already occurs at low densities, harvesting may have a large effect on the potential fertilization success of geoduck. However, little is known of the density dependence of fertilization success for geoduck species and this warrants further investigation. Further to this, *P. zelandica* is protandric with females dominating the large size classes (Gribben & Creese 2003). Given that harvesting geoduck involves searching for siphon holes and the largest siphon holes generally contain the largest geoduck, harvesting may also target female geoduck resulting in populations that are egg-limited (Gribben & Creese 2003). This may have severe repercussions for future recruitment success.

TABLE 2.

Estimates of total area, show-factor (SF) adjusted total, mean density, total abundance, mean individual weight and biomass of geoduck found in all transects in each location.

	Area (ha)	SF Adj. Total (\pm SE)	Density (m^{-2}) (\pm SE)	Abundance (\pm SE)	Mean Weight (g) (\pm SE)	Biomass (t) (\pm SE)
KB	39.60	382.9 (131.5)	0.06 (0.01)	22976.0 (4007.3)	242.2 (8.6)	5.56 (0.99)
SB	1.87	457.3 (131.5)	0.49 (0.08)	9146.6 (1421.2)	358.8 (7.5)	3.28 (0.51)
MB	2.17	148.8 (34.3)	0.14 (0.01)	2975.9 (282.1)	358.8 (7.5)	1.07 (0.10)
B1	0.47	91.9 (63.57)	0.39 (0.17)	1838.1 (803.7)	358.8 (7.5)	0.66 (0.29)
B2	0.59	224.4 (181.9)	0.45 (0.11)	2669.6 (637.2)	358.8 (7.5)	0.96 (0.23)

KB, Kennedy Bay; SB, Shelly Bay; MB, Mahunga Bay; B1, Bay 1, and B2, Bay 2.
Standard error (\pm SE) given in parentheses.

The large differences in mean density and size of geoduck between the Kennedy Bay and Wellington populations may, in part, be due to large storm events that occurred in the Coromandel region in the mid 1990s, which saw large numbers of geoduck stranded on the beaches surrounding Kennedy Bay (George Potae, Kennedy Bay Mussel Company Ltd. pers. comm.). Since then, a large recruitment event occurred during 1997/1998 resulting in the population being dominated by two cohorts (Gribben et al. 2004). Geoduck in Shelly Bay were mainly large individuals with very few small geoduck present. Thus mean size and biomass in Kennedy Bay are unlikely to be static and will increase as the cohort develops. The future contribution that this smaller cohort will make to later biomass estimates is unknown because no reliable estimates of mortality are currently available for *P. zelandica* (Breen 1991, Gribben 2003).

The results presented in this study indicate that the methods used for describing the distribution, density, and biomass of populations of *P. abrupta* could be readily adopted for *P. zelandica*. However, less experienced searchers may have more difficulty in finding geoduck, especially when visibility is poor and during

winter months. During this period, geoduck were usually found by searching potential siphon holes, which is more time consuming. We recommend that abundance and biomass estimates not be conducted during winter or periods of poor visibility because less experienced divers may miss many geoduck leading to biomass underestimates, and that the variability in diver counts be investigated. Finally, before the sustainability of harvesting geoduck can be assessed further, work on the density dependence of fertilization success, recruitment, and natural mortality of *P. zelandica* must be conducted.

ACKNOWLEDGMENTS

The authors thank Robert Williamson, George Potae, and Andrew Bell for their assistance in collecting geoduck and Drs. Jonathan Gardner and Bob Wear for use of equipment. Thanks also to two reviewers, Drs. Alan Campbell and Wayne Hajas, for their helpful comments and suggestions on an earlier version of this manuscript. This study was supported by a Graduate Research in Industry Foundation scholarship, made possible with the help of the members of the Geoduck Research Group.

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MICROSATELLITE AND ALLOZYME ANALYSES REVEAL FEW GENETIC DIFFERENCES AMONG SPATIALLY DISTINCT AGGREGATIONS OF GEODUCK CLAMS (*PANOPEA ABRUPTA*, CONRAD 1849)

BRENT VADOPALAS,^{1*} LARRY L. LECLAIR² AND PAUL BENTZEN³

¹School of Aquatic and Fishery Sciences, University of Washington, Box 355020, Seattle, Washington 98195; ²Washington Department of Fish and Wildlife, 600 Capitol Way N, Olympia, Washington 98501; ³Dept. of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4J1

ABSTRACT The genetic population structure of geoduck clams (*Panopea abrupta*) in inland waters of Washington may affect fishery management and aquacultural practices involving this species. To investigate genetic differentiation in geoduck clams, samples were collected from 16 Washington State sites located in the five Puget Sound sub basins, southern Georgia Strait, and the Strait of Juan de Fuca. A collection from Clarence Strait in SE Alaska was included as an outgroup. Individuals were genotyped at 11 allozyme and 7 microsatellite loci. There was little differentiation overall, but the Freshwater Bay collection in the Strait of Juan de Fuca was differentiated from others at both microsatellite and allozyme loci. For both marker classes, there was no evidence of significant correlation between genetic and geographic distance measures. In contrast to the microsatellite loci, the allozyme loci were in Hardy-Weinberg Equilibrium (HWE). Deviations from HWE expectations at microsatellite loci were interpreted as being primarily due to primer-site sequence variation rather than population level processes such as inbreeding.

KEY WORDS: *Panopea abrupta*, geoduck, population genetics, larval dispersal

INTRODUCTION

Understanding the extent of gene flow provides insight into the demographic dynamics among natural populations. Generally, gene flow is correlated with dispersal ability in many organisms (Bohonak 1999), including many marine fishes and shellfishes (reviewed in Shaklee & Bentzen 1998). In sedentary marine bivalves, dispersal and gene flow occur only during the pelagic larval phase. Gene flow and larval dispersal may be correlated with spatial distribution in marine mollusks (Johnson et al. 2001); many investigators have failed to falsify the null hypothesis of panmixia at broad geographic scales in a variety of broadcast spawning marine species with pelagic larvae (e.g., *Crassostrea virginica* [McDonald et al. 1996]; *Littorina striata*, [De Wolf et al. 2000]; *Mytilus galloprovincialis* [Skalamera et al. 1999]). Nevertheless, some studies have demonstrated genetic structuring in a variety of marine invertebrate species with pelagic larvae (e.g., the American oyster, *Crassostrea virginica* [Reeb & Avise 1990]; *Ostrea edulis* [Launey et al. 2002]; the sea urchins *Strongylocentrotus purpuratus* [Edmands et al. 1996] and *S. franciscanus* [Moberg & Burton 2000]; and black abalone *Haliotis cracherodii* [Hamm & Burton 2000]). Examples of genetic differentiation on smaller geographic scales include the limpet *Siphonaria jeanae* (Johnson & Black 1984), the oyster *Crassostrea virginica* (Buroker 1983, [King et al. 1994]), cockles *Cerastoderma glaucum* (Mariani et al. 2002), and *Mytilus edulis* (Ridgway 2001). Most population genetic studies of marine invertebrates have focused on populations that are distributed along open coasts or island populations with discontinuous distributions separated by deep oceanic water. However, Parsons (1996) demonstrated significant genetic subdivision (9-locus F_{ST} = 0.16) over an area of only 75 km² in the intertidal gastropod, *Austrocochlea constricta*. Limited water movement in the area studied may have caused localized recruitment, resulting in the detected population differentiation (Parsons 1996).

The complex hydrology and bathymetry of Puget Sound in Washington State suggests the potential for restricted dispersal and population subdivision of marine invertebrates in the region. Puget Sound is a fjord-like estuarine system that is highly subdivided by numerous peninsulas, narrow passes, islands, and underwater sills (Fig. 1). It is comprised of five sub-basins oriented approximately along a north-south axis. At the north end is Admiralty Inlet, separated from the Strait of Juan de Fuca by a shallow sill. Admiralty Inlet connects to three long, narrow basins: from west to east these are Hood Canal, Main Basin, and Whidbey Basin. Southern Basin is connected at the south end of the Main Basin. Potentially important for larval dispersal, each basin is separated from its neighbor by a shallow sill, except for Whidbey Basin's constricted connection to the Main Basin (Ebbesmeyer et al. 1988). The Strait of Juan de Fuca, the sole connection of Puget Sound to the Pacific Ocean, is itself bounded by an inner and an outer sill, as well as the bathymetrically and hydrologically complex Georgia Strait to the north (Herlinveaux & Tully 1961). Collectively, these features restrict the flow of surface waters, leading to the possibility that the dispersal of pelagic larvae may be restricted, and hence that genetic differentiation of marine invertebrates might occur on relatively small geographic scales. On the other hand, Puget Sound's freshwater inputs and their typical estuarine surface outflow increase the propensity of passive surface particles to disperse in a seaward direction, and could be sufficient to genetically homogenize molluscan populations colonized by pelagic larvae drifting seaward from populations in inner inlets. In a study of marine bivalve population genetics in Puget Sound, Parker et al. (2003) recently found significant differences in allele frequencies among collections of the endemic *Prototheca staminea*, but no differences among collections of *Macoma balthica*.

One of the most fecund species with pelagic larvae in Puget Sound, Washington, the bivalve *Panopea abrupta*, or geoduck clam, is found there in high densities and is among the largest, longest-lived (> 100 y), and deepest burrowing clams known. It occurs from the low intertidal zone to a depth of 100 m, buried up

*Corresponding author. brentv@u.washington.edu

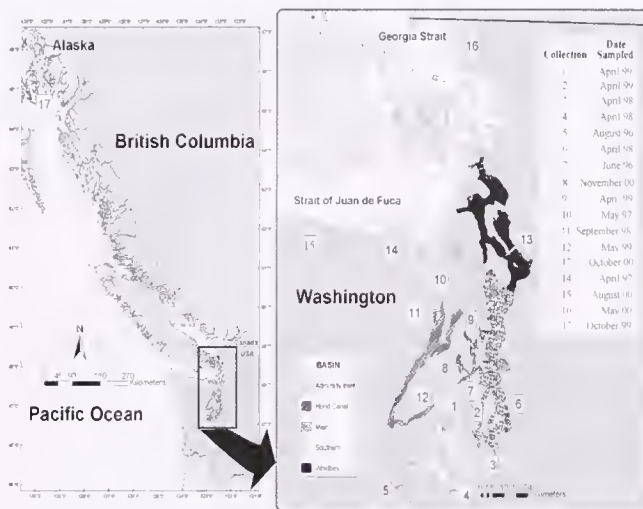


Figure 1. Puget Sound hydrographic basins, and sample dates and geographic locations for 17 geoduck clam (*Panopea abrupta*) collections from Washington and Southeast Alaska.

to 1 m in loose stable substrates in marine and estuarine environments from Alaska to Mexico (Goodwin & Pease 1989). Geoducks are dioecious synchronized broadcast spawners, with a pelagic larval phase that lasts from 3–6 weeks (Goodwin 1976). Experimental evidence indicates mean fecundities of 40 million eggs per year (Beattie 1992). A species may use the strategy of high fecundity to overcome the spatial and temporal odds of gamete union (Hedgecock 1994) and/or larval survival, or, secondarily, to maximize dispersal (Johnson et al. 2001).

Geoducks are the target of a lucrative international commercial fishery, and recently have become a commercially cultivated species. Assessing potential impacts of hatchery outplants on their wild counterparts will depend, in part, on the characterization of

naturally occurring genetic stock structure. To date there has been no available information on the genetic stock structure of *P. abrupta*, or any of its congeners, and with the high fecundity and longevity of *P. abrupta* potentially maximizing dispersal and genetic homogeneity, large-scale panmixia has been the assumption. Here, we evaluate the null hypothesis of genetic homogeneity in the Strait of Juan de Fuca—Georgia Strait—Puget Sound complex. We examine population differentiation among collections from sites in the complex using 11 allozyme and seven microsatellite loci. As alternative hypotheses, we also consider genetic differentiation due to isolation by distance, as well as stochastic genetic differentiation relative to geographic distance.

A secondary objective is to compare the outcome of parallel analyses using two classes of genetic marker, allozymes and microsatellites. Both are codominant, however allelic variability is usually much higher in microsatellites, providing potentially higher power to detect differences (Goudet et al. 1996, Grant et al. 2000; but see Allendorf & Seeb 2000 and DeWoody & Avise 2000). Because discordant results have been achieved with the two marker classes in a number of studies of genetic variation (e.g., Lemaire et al. 2000, de Innocentiis et al. 2001 but see Allendorf & Seeb 2000), we investigate the relative strengths of these two marker classes for detection of genetic differentiation in a broadcast spawning marine bivalve.

METHODS

Sample Collection

Using SCUBA, divers collected geoducks ($n = 1645$) from a total of 17 sites distributed among the five sub-basins of Puget Sound, the Strait of Juan de Fuca, Georgia Strait, and near Ketchikan, Alaska (Fig. 1). Where possible, sites were chosen based on maximum watercourse distances, least amount of surface water outflow, maximum potential wind-mediated retention, and geographic coverage. Tissue samples were dissected from live animals within 24 hours of harvest. Animals that were not dissected immediately after harvest were held live in flow-through troughs

TABLE 1.

Pairwise watercourse distances (upper triangle) and F_{st} values (allozyme/microsatellite) for 17 collections of *Panopea abrupta* from Puget Sound, Washington and S.E. Alaska. Bold type indicates significant pairwise tests of genotypic (7 microsatellite loci) and genic (11 allozyme loci) differentiation; underlining indicates significant pairwise tests of genotypic differentiation for all 18 loci combined. Indicated significance is at the table-wide Bonferroni P -value of 0.00028 (0.05/178).

	1	2	3	4	5	6	7	8
1		50	19	29	7	93	99	130
2	/0.0017		44	34	35	56	70	92
3	-0.0012/	/		27	35	37	54	74
4	-0.0026/	/	-0.0005/		19	52	68	88
5	0.0049/	/	0.0020/	0.0034/		83	92	120
6	0.0004/0.0007	/0.0016	0.0008/	0.0019/	0.0024/		17	37
7	0.0012/	/	0.0023/	-0.0007/	0.0001/	0.0013/		20
8	0.0071/-0.0007	/0.0007	0.0049/	0.0078/	0.0021/	0.0009/0.0000	0.0050/	
9	0.0000/	/	0.0018/	0.0014/	0.0054/	0.0039/	-0.0032/	0.0128/
10	0.0039/	/	0.0037/	0.0053/	-0.0011/	0.0002/	0.0012/	-0.0008/
11	0.0024/-0.0003	/0.0007	0.0007/	0.0027/	0.0003/	0.0021/0.0011	0.0035/	<u>-0.0001/-0.0004</u>
12	0.0007/0.0010	/0.0016	0.0006/	0.0027/	0.0014/	0.0007/0.0014	-0.0008/	<u>0.0028/0.0000</u>
13	0.0053/0.0003	/0.0028	0.0040/	0.0049/	0.0008/	-0.0013/-0.0004	0.0013/	-0.0015/-0.0004
14	0.0014/	/	0.0035/	0.0016/	0.0042/	0.0026/	0.0001/	0.0027/
15	0.0081/0.0022	/0.0033	0.0097/	0.0068/	0.0024/	<u>0.0020/0.0031</u>	0.0025/	<u>0.0068/0.0028</u>
16	0.0003/0.0019	/0.0005	0.0019/	0.0039/	0.0058/	-0.0012/0.0007	0.0072/	<u>-0.0002/-0.0005</u>
17	/0.0016	/0.0027	/	/	/	/0.0003	/	/0.0004

supplied with natural seawater. From each specimen, samples were taken for allozyme, microsatellite, or both analyses: approximately 1 cm³ samples of siphon and ctenidia tissues were collected and cryogenically preserved at -80 °C, and foot tissues were divided into 0.5 cm pieces, and stored in 95% ethanol at room temperature (DNA) until laboratory analyses. Sampling the SE Alaska specimens for allozyme analyses was not possible because tissues for microsatellite analyses were obtained opportunistically via a commercial harvest.

Microsatellite Data Collection

Microsatellite genotypes were collected from specimens from sites 1, 2, 6, 8, 11, 12, 13, 15, 16, and 17 (Fig. 1). DNA was extracted from 10 mg of foot tissue using DNEasy 96 Tissue Kits (Qiagen) following the manufacturer's protocol, and used to amplify seven loci. Four of these loci, Pab3, Pab4, Pab5, and Pab6 (Genbank accession numbers AF213657–AF213660) were described by Vadopalas and Bentzen (2000). For Pab5, the primer TAATTCAGGTGGCCGATTT was used as an alternate forward primer. Pab7 (CA repeat, 137–199 bp, primers TTTTCAACTG-GATTGCGTGA and GAACCAATCAATAGAAGCTCCA), Pab8 (compound tetranucleotide repeat, 200–634 bp, primers TCAATGAGATAAAAATGTCGCTAAC and AACAATACCTGCACCAATCT), and Pab9 (GATA repeat, 202–300 bp, primers CGTAAATGTTTATGCCTGCAA and GATCACAAC-TCTCTTTTCTTC) reported here for the first time, were isolated as described in Vadopalas and Bentzen (2000) (Genbank accession numbers AF541256–AF541258). One of each primer pair was labeled on the 5' end with NED (Pab4, Pab8), FAM (Pab5, Pab6, Pab7), or HEX (Pab1, Pab9) fluorescent dyes to enable detection on the MegaBACE 1000 genotyping platform. Reactions were carried out in 384 well microtitre plates in 10 µL volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01 µM BSA, 0.1% Triton-X100, 0.2 mM each dNTP, 0.5 µM each primer, 0.5 U *Taq* DNA polymerase, and 25–100 ng genomic DNA. Cycling conditions were as follows: 95 °C (3 min); five cycles 95 °C (30 s), 52 °C (30 s) and 72 °C (30 s); 25 cycles 90 °C (15 s), 52 °C (15 s), 72 °C (30 s); 72 °C (40 min) final extension.

Loci were amplified individually and then pooled in sets of four according to nonoverlapping allelic ranges and differing fluorescent end labels. The two electrophoresis multiplexes consisted of Pab3-Pab5-Pab8 and Pab4-Pab6-Pab7-Pab9, and were desalted using hydrated Sephadex G-75 (medium) in Millipore Multiscreen HV 0.45 µm filter plates. Some PCR products were treated with Precipitate (CPG, Inc. New Jersey) following the manufacturer's protocol prior to the desalting step to prolong MegaBACE capillary life. Prepared samples were further diluted 2–5 times, and 2.05 µL added to 2.75 µL MegaBACE loading buffer (Amersham), plus 0.2 µL 900 bp internal size standard (25 bp rungs, Amersham). Pooled samples were denatured in a 96-well heat block at 95 °C for 55 sec and snap-cooled in a 96-well cold block for 1.5 min before electrophoresis through a 96 well MegaBACE 1000 capillary system. Allele sizes were estimated by visual inspection of raw electrophoretic traces using Genotyper 1.0 software (Amersham) and subsequently binned by grouping to the nearest full repeat.

Allozyme Data Collection

Preliminary allozyme screening included over 30 enzymes and a wide array of tissue-buffer combinations. Based on those results, an 11-enzyme, 2-tissue (siphon muscle and ctenidium) screening protocol was developed and used for all subsequent assays. The following enzyme/buffer combinations were used: alanine aminotransferase (ALAT), arginine kinase (ARGK), cytosol nonspecific dipeptidase (PEPA), mannose-6-phosphate isomerase (MPI), and superoxide dismutase (SOD) on TRIS-GLY pH 8.5 (Holmes & Masters 1970); aspartate aminotransferase (AAT-1), glucose-6-phosphate isomerase (GPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and phosphogluconate dehydrogenase (PGDH) on CAME pH 6.8 (Clayton & Tretiak 1972); isocitrate dehydrogenase (IDHP), malate dehydrogenase 9 (MDH), and the *SOD** 155 allele on CAME pH 5.9. The histochemical staining recipes of Harris and Hopkinson (1976) were used to visualize all allozymes except SOD, which was stained according to Fevolden (1989).

For each locus, variant alleles are described in terms of the mobility of the encoded allozymes, relative to the mobility of the

TABLE 1.
continued

	9	10	11	12	13	14	15	16	17
1	145	155	217	249	158	199	244	258	1270
2	109	130	192	224	132	180	219	233	1245
3	91	101	174	206	114	155	200	215	1227
4	105	115	188	220	129	170	215	229	1241
5	138	148	210	242	151	192	237	251	1273
6	54	64	137	169	77	118	163	178	1190
7	50	60	135	167	75	116	161	176	1173
8	57	67	140	172	80	121	166	181	1149
9		15	73	105	34	54	99	113	1126
10	0.0062/		63	95	44	44	89	103	1116
11	0.0066/	0.0008/		65	107	107	152	166	1178
12	0.0010/	-0.0003/	0.0044/-0.0003		139	139	184	198	1210
13	0.0057/	-0.0003/	0.0004/0.0002	0.0028/0.0006		88	133	147	1159
14	0.0048/	0.0021/	0.0008/	0.0026/	0.0022/		45	69	1171
15	0.0069/	0.0021/	0.0055/0.0009	0.0080/0.0023	0.0022/0.0021	0.0088/		114	1126
16	0.0094/	0.0012/	0.0012/0.0002	0.0025/0.0004	0.0021/0.0015	0.0012/	0.0102/0.0018		1012
17	/	/	/0.000	/0.0007	/0.0000	/	/-0.0007	/0.0004	

TABLE 2.

Allelic variability in 15 populations of *Panopea abrupta* at 11 allozyme loci. Shown for each population and locus: number of alleles (#), expected heterozygosity (H_e), observed heterozygosity (H_o), p -value for test of conformance to HWE (p), within-population variability (F_{is}), and number of individuals genotyped (n). Means are given in the last column.

		Locus											
Collection		ALAT	MPI	PEPA	PGDH	SOD	ARGK	GPI	AATI	GAPDH	MDH	IDHP	Means
1	#	3	8	4	4	4	4	4	5	2	2	3	3.909
	H _e	0.138	0.755	0.484	0.031	0.477	0.568	0.589	0.258	0.041	0.481	0.497	0.392
	H _o	0.135	0.750	0.406	0.032	0.448	0.635	0.635	0.250	0.042	0.438	0.406	0.380
	p	0.201	0.775	0.041	1.000	0.838	0.063	0.787	0.115	1.000	0.399	0.073	0.180
	F _{is}	0.017	0.006	0.160	-0.005	0.062	-0.119	-0.080	0.032	-0.016	0.090	0.182	0.016
	n	103	103	103	102	103	103	103	103	103	103	103	
3	#	4	7	3	5	4	4	5	5	2	2	4	4.091
	H _e	0.121	0.746	0.451	0.046	0.464	0.594	0.547	0.176	0.044	0.491	0.516	0.381
	H _o	0.127	0.773	0.509	0.047	0.482	0.573	0.527	0.173	0.046	0.518	0.518	0.390
	p	1.000	0.000	0.384	1.000	0.909	0.826	0.759	0.599	1.000	0.697	0.373	0.279
	F _{is}	-0.051	-0.031	-0.125	-0.010	-0.033	0.040	0.041	0.021	-0.019	-0.051	0.000	-0.019
	n	110	110	110	107	110	110	110	110	110	110	110	
4	#	4	8	4	7	5	7	3	6	2	3	5	4.909
	H _e	0.198	0.754	0.455	0.074	0.474	0.592	0.570	0.204	0.017	0.501	0.500	0.394
	H _o	0.208	0.658	0.483	0.076	0.417	0.617	0.525	0.175	0.017	0.542	0.467	0.380
	p	0.221	0.038	0.518	1.000	0.534	0.159	0.487	0.421	1.000	0.003	0.664	0.085
	F _{is}	-0.050	0.131	-0.058	-0.019	0.126	-0.038	0.084	0.145	-0.004	-0.076	0.070	0.040
	n	120	120	120	119	120	120	120	120	120	120	120	
5	#	4	8	3	5	4	5	4	4	2	3	4	4.182
	H _e	0.198	0.757	0.362	0.069	0.426	0.588	0.582	0.177	0.068	0.495	0.501	0.384
	H _o	0.202	0.675	0.395	0.070	0.377	0.553	0.711	0.175	0.070	0.483	0.500	0.383
	p	1.000	0.085	0.604	1.000	0.509	0.201	0.025	0.674	1.000	0.912	1.000	0.652
	F _{is}	-0.019	0.108	-0.089	-0.020	0.114	0.059	-0.220	0.011	-0.032	0.025	0.002	0.003
	n	114	114	114	114	114	114	114	114	114	114	114	
6	#	3	8	3	6	3	4	5	3	2	3	4	4.000
	H _e	0.199	0.712	0.443	0.076	0.506	0.607	0.590	0.247	0.032	0.490	0.514	0.401
	H _o	0.185	0.706	0.380	0.077	0.554	0.663	0.576	0.261	0.033	0.435	0.446	0.392
	p	0.290	0.679	0.140	1.000	0.637	0.294	0.036	1.000	1.000	0.230	0.212	0.380
	F _{is}	0.070	0.008	0.142	-0.019	-0.095	-0.092	0.023	-0.057	-0.011	0.113	0.133	0.023
	n	92	92	92	91	92	92	92	92	92	92	92	
7	#	4	6	3	4	4	5	4	4	2	3	3	3.818
	H _e	0.252	0.741	0.443	0.053	0.493	0.627	0.588	0.182	0.069	0.510	0.471	0.403
	H _o	0.268	0.661	0.429	0.054	0.464	0.661	0.607	0.196	0.071	0.536	0.339	0.390
	p	0.270	0.116	0.222	1.000	0.867	0.147	0.182	1.000	1.000	0.886	0.051	0.364
	F _{is}	-0.065	0.108	0.032	-0.009	0.058	-0.054	-0.032	-0.081	-0.028	-0.051	0.280	0.032
	n	56	56	56	56	56	56	56	56	56	56	56	
8	#	3	8	4	2	5	6	4	5	3	2	3	4.091
	H _e	0.180	0.709	0.361	0.020	0.427	0.632	0.595	0.165	0.076	0.459	0.508	0.376
	H _o	0.176	0.647	0.314	0.020	0.422	0.549	0.598	0.157	0.078	0.412	0.451	0.348
	p	0.644	0.216	0.313	1.000	0.481	0.020	0.939	0.534	1.000	0.385	0.276	0.495
	F _{is}	0.021	0.087	0.013	-0.005	0.012	0.131	-0.005	0.051	-0.028	0.103	0.112	0.074
	n	102	102	102	99	102	102	102	102	102	102	102	
9	#	2	7	3	4	3	3	4	2	2	3	4	3.364
	H _e	0.130	0.757	0.510	0.064	0.455	0.570	0.568	0.226	0.058	0.506	0.462	0.391
	H _o	0.140	0.640	0.460	0.065	0.520	0.640	0.540	0.220	0.060	0.600	0.360	0.386
	p	1.000	0.010	0.047	1.000	0.681	0.450	0.770	1.000	1.000	0.252	0.060	0.223
	F _{is}	-0.065	0.165	0.107	-0.011	-0.132	-0.114	0.060	0.038	-0.021	-0.176	0.230	0.024
	n	50	50	50	46	50	50	50	50	50	50	50	
10	#	5	8	4	5	4	4	3	4	3	4	4	4.364
	H _e	0.244	0.717	0.393	0.033	0.419	0.598	0.580	0.203	0.057	0.479	0.501	0.384
	H _o	0.208	0.658	0.392	0.033	0.392	0.558	0.542	0.192	0.058	0.467	0.483	0.362
	p	0.076	0.219	0.834	1.000	0.599	0.276	0.094	0.549	1.000	0.001	0.909	0.085
	F _{is}	0.150	0.086	0.007	-0.006	0.069	0.070	0.070	0.060	-0.022	0.031	0.038	0.061
	n	120	120	120	120	120	120	120	120	120	120	120	

continued on next page

TABLE 2.
continued

Collection	Locus											Means
	ALAT	MPI	PEPA	PGDH	SOD	ARGK	GPI	AATI	GAPDH	MDH	IDHP	
11 #	3	9	4	4	5	6	4	5	3	2	2	4.273
H _c	0.155	0.764	0.413	0.031	0.445	0.589	0.627	0.175	0.071	0.469	0.500	0.385
H _o	0.125	0.802	0.375	0.031	0.521	0.500	0.677	0.188	0.073	0.448	0.448	0.381
p	0.122	0.079	0.214	1.000	0.170	0.128	0.486	1.000	0.107	0.668	0.411	0.159
F _{is}	0.195	-0.049	0.092	-0.005	-0.169	0.151	-0.080	-0.070	-0.028	0.044	0.105	0.010
n	99	99	99	99	99	99	99	99	99	99	99	
12 #	3	8	4	4	5	6	3	4	2	2	5	4.182
H _c	0.137	0.716	0.437	0.043	0.440	0.618	0.539	0.247	0.010	0.478	0.507	0.379
H _o	0.146	0.590	0.396	0.043	0.385	0.600	0.531	0.271	0.010	0.510	0.531	0.365
p	1.000	0.015	0.122	1.000	0.038	0.547	0.975	0.247	1.000	0.528	0.806	0.204
F _{is}	-0.068	0.177	0.094	-0.010	0.124	0.029	0.015	-0.097	0.000	-0.067	-0.047	0.038
n	100	99	100	97	100	99	100	100	100	100	100	
13 #	3	6	3	5	4	6	4	3	3	3	4	4.000
H _c	0.213	0.733	0.358	0.072	0.477	0.597	0.613	0.189	0.041	0.501	0.510	0.391
H _o	0.240	0.625	0.406	0.074	0.500	0.677	0.677	0.208	0.042	0.458	0.448	0.396
p	0.651	0.069	0.428	1.000	0.948	0.022	0.279	1.000	1.000	0.413	0.258	0.423
F _{is}	-0.125	0.148	-0.136	-0.019	-0.048	-0.134	-0.105	-0.100	-0.012	0.085	0.121	-0.005
n	117	117	117	117	117	117	117	117	117	117	117	
14 #	4	8	4	6	4	4	5	4	2	5	4	4.545
H _c	0.247	0.753	0.423	0.041	0.472	0.608	0.592	0.232	0.013	0.469	0.493	0.395
H _o	0.210	0.676	0.442	0.041	0.435	0.655	0.601	0.237	0.014	0.426	0.493	0.385
p	0.044	0.012	0.494	1.000	0.043	0.260	0.136	1.000	1.000	0.435	0.351	0.058
F _{is}	0.153	0.103	-0.045	-0.009	0.078	-0.077	-0.017	-0.019	-0.003	0.092	0.000	0.026
n	148	148	147	146	147	148	148	148	148	148	148	
15 #	5	8	3	3	3	5	4	5	2	2	4	4.000
H _c	0.200	0.741	0.402	0.042	0.477	0.628	0.572	0.191	0.031	0.479	0.499	0.387
H _o	0.198	0.649	0.354	0.042	0.542	0.667	0.663	0.156	0.031	0.427	0.432	0.378
p	0.701	0.078	0.393	1.000	0.187	0.096	0.209	0.069	1.000	0.390	0.199	0.136
F _{is}	0.010	0.124	0.119	-0.012	-0.136	-0.061	-0.160	0.181	-0.011	0.108	0.135	0.018
n	105	103	105	104	105	105	104	105	105	105	103	
16 #	4	8	4	3	3	5	5	3	3	3	4	4.091
H _c	0.211	0.738	0.456	0.022	0.446	0.592	0.583	0.232	0.052	0.449	0.518	0.391
H _o	0.158	0.684	0.474	0.022	0.358	0.547	0.484	0.211	0.053	0.411	0.442	0.349
p	0.038	0.539	0.764	1.000	0.090	0.734	0.049	0.172	1.000	0.652	0.110	0.157
F _{is}	0.252	0.073	-0.040	-0.003	0.197	0.075	0.169	0.094	-0.017	0.086	0.147	0.104
n	98	98	98	93	98	98	98	98	98	98	98	
total #	7	9	5	11	7	8	6	8	4	6	9	7.27
p	0.1879	<0.0001	0.1719	1.0000	0.3496	0.0051	0.0849	0.7546	1.0000	0.0907	0.1801	<0.0001

most frequently encountered allele during the preliminary survey. Allozyme genotypes were collected from the same sites noted earlier, except for sites 2 and 17. Additional collections analyzed for allozyme variation only included three samples from the Southern Basin, (sites 3, 4, and 5), two samples from the Main Basin (7 and 9), and one sample from Admiralty Inlet (10).

Data Analyses

Genepop version 3.3 population genetics software of Raymond and Rousset (1995) was used to test for conformation to Hardy-Weinberg Equilibrium (HWE) at each locus in each collection using the Markov chain exact test method (Guo & Thompson 1992) for loci with five or more alleles, and the Louis and Dempster (1987) enumeration method for loci with less than five alleles (allozyme loci *PEPA**, *GAPDH**, and *MDH**). Genepop was also used to estimate gametic linkage disequilibrium between loci via the algorithm described by Cockerham and Weir (1979). For

analyses of population differentiation, the genotypic log-likelihood (g) based exact test (Goudet et al. 1996), as implemented in Genepop, was used for all loci. To increase power in groups of loci in which assumptions of HWE were not violated, the genic test (Raymond & Rousset 1995) was used as implemented in Genepop. Weir and Cockerham's (1984) unbiased estimators of single and multilocus F-statistics were computed using the program FSTAT version 2.9.3.2 (Goudet 1995), and F_{ST} values were jackknifed over loci, and bootstrapped over individuals to obtain 95% confidence limits. The programs described in Beaumont and Nichols (1996) were used to evaluate whether F_{ST} values might indicate spatial selection at individual loci. Subsets of the allozyme and microsatellite datasets were used to perform a hierarchical analyses of molecular variance (AMOVA, Excoffier et al. 1992) based on the infinite allele model, to partition the genetic variance into among basins, among collections within basins, and within collections. The significance of the fixation indices among and

TABLE 3.

Allelic variability in 10 populations of *Panopea abrupta* at 7 microsatellite loci. Shown for each population and locus: number of alleles (#), expected heterozygosity (H_e), observed heterozygosity (H_o), p-value for test of conformance to HWE (p), within-population variability (F_{is}), and number of individuals genotyped (n). Means are given in the last column.

Collection		Locus							Mean
		Pab3	Pab4	Pab5	Pab6	Pab7	Pab8	Pab9	
1	#	31	40	19	31	16	60	20	31
	H_e	0.95	0.97	0.89	0.93	0.91	0.98	0.93	0.93
	H_o	0.48	0.33	0.53	0.91	0.73	0.51	0.80	0.61
	p	<.0001	<.0001	<.0001	0.253	0.000	0.001	0.011	<.0001
	F_{is}	0.491	0.655	0.406	0.018	0.198	0.486	0.137	0.345
	n	95	90	95	92	91	95	90	
2	#	26	33	21	28	19	49	19	27.86
	H_e	0.94	0.96	0.90	0.93	0.91	0.98	0.92	0.93
	H_o	0.50	0.43	0.50	0.89	0.57	0.52	0.74	0.59
	p	<.0001	<.0001	<.0001	0.563	<.0001	<.0001	<.0001	<.0001
	F_{is}	0.472	0.547	0.447	0.048	0.081	0.467	0.197	0.365
	n	96	92	96	96	96	96	96	
6	#	26	31	18	29	15	59	18	28
	H_e	0.96	0.97	0.91	0.92	0.88	0.99	0.92	0.94
	H_o	0.52	0.28	0.42	0.85	0.59	0.41	0.75	0.55
	p	<.0001	<.0001	<.0001	0.052	<.0001	<.0001	<.0001	<.0001
	F_{is}	0.454	0.713	0.536	0.080	0.332	0.580	0.191	0.417
	n	92	83	92	92	87	87	91	
8	#	31	28	21	28	20	56	18	28.857
	H_e	0.96	0.97	0.90	0.93	0.92	0.98	0.92	0.94
	H_o	0.53	0.33	0.63	0.88	0.82	0.44	0.83	0.64
	p	<.0001	<.0001	<.0001	0.241	<.0001	0.055	0.026	<.0001
	F_{is}	0.444	0.664	0.304	0.049	0.113	0.551	0.019	0.323
	n	96	89	96	95	92	91	95	
11	#	28	32	25	31	17	52	19	29.14
	H_e	0.95	0.96	0.92	0.94	0.91	0.98	0.93	0.94
	H_o	0.49	0.43	0.44	0.90	0.53	0.44	0.79	0.57
	p	<.0001	<.0001	<.0001	0.107	<.0001	<.0001	0.074	<.0001
	F_{is}	0.484	0.551	0.525	0.044	0.417	0.552	0.145	0.390
	n	96	81	96	96	96	96	96	
12	#	28	31	19	26	18	52	17	27.29
	H_e	0.96	0.96	0.91	0.93	0.91	0.98	0.92	0.94
	H_o	0.45	0.31	0.45	0.87	0.60	0.45	0.75	0.55
	p	<.0001	<.0001	<.0001	0.163	<.0001	<.0001	0.004	<.0001
	F_{is}	0.533	0.673	0.506	0.067	0.339	0.546	0.182	0.410
	n	94	89	94	91	96	92	92	93
13	#	30	32	19	29	15	53	18	28
	H_e	0.96	0.96	0.90	0.93	0.90	0.98	0.92	0.93
	H_o	0.26	0.33	0.49	0.95	0.61	0.43	0.79	0.55
	p	<.0001	<.0001	<.0001	0.696	<.0001	<.0001	<.0001	<.0001
	F_{is}	0.728	0.657	0.459	-0.024	0.321	0.566	0.141	0.411
	n	96	79	96	96	96	94	96	
15	#	28	35	21	24	19	67	18	30.29
	H_e	0.95	0.96	0.93	0.93	0.91	0.99	0.93	0.94
	H_o	0.44	0.31	0.57	0.89	0.84	0.39	0.81	0.61
	p	<.0001	<.0001	<.0001	0.284	0.010	0.002	0.047	<.0001
	F_{is}	0.535	0.675	0.385	0.038	0.081	0.601	0.134	0.355
	n	95	83	95	93	94	94	93	
16	#	31	37	19	34	17	52	21	30.14
	H_e	0.95	0.97	0.92	0.94	0.91	0.98	0.93	0.94
	H_o	0.71	0.35	0.63	0.87	0.72	0.81	0.81	0.7
	p	<.0001	<.0001	<.0001	0.017	<.0001	<.0001	0.0213	<.0001
	F_{is}	0.252	0.642	0.321	0.07	0.208	0.168	0.124	0.257
	n	94	89	96	92	96	96	96	
17	#	31	34	19	29	17	58	21	29.86
	H_e	0.95	0.96	0.92	0.93	0.91	0.98	0.93	0.94
	H_o	0.53	0.34	0.48	0.87	0.64	0.44	0.84	0.59
	p	<.0001	<.0001	<.0001	0.2219	<.0001	<.0001	0.2845	<.0001
	F_{is}	0.443	0.647	0.482	0.061	0.305	0.554	0.098	0.373
	n	94	88	96	93	96	91	94	
total #		40	50	29	50	25	96	31	
P		<.0001	<.0001	<.0001	0.078	<.0001	<.0001	<.0001	

within basins and among samples was tested by nonparametric genotypic permutation tests as implemented in Arlequin (Schneider et al. 2000). Two distance matrices, one using linear watercourse distances (Table 1), the other with distances weighted by average subbasin retention time (Ebbesmeyer et al. 1988) between each pair of samples, were tested for correlation with genetic distance matrices via Mantel tests (Mantel 1967) using rank correlations (Raymond & Rousset 1995). A significance level of 0.05 was used throughout, and to avoid Type I error the Bonferroni correction (Rice 1989) was applied for table-wide comparisons.

RESULTS

Within-Population Genetic Diversity

As expected, levels of genetic diversity exhibited by the microsatellites were greater than those observed at allozyme loci (Tables 2 and 3). All of the microsatellites, but only 11 of 30 allozyme loci assayed were polymorphic. The mean number of alleles per allozyme locus per sample was 3.3 to 4.5 (overall mean 4.1), compared with 27.3 to 31.0 (overall mean 29.9) for the microsatellite loci. Expected heterozygosities were 0.01 to 0.76 (mean 0.39) for allozyme loci and 0.88 to 0.99 (mean 0.94) for microsatellites. No significant linkage disequilibrium was detected between pairs of allozyme or microsatellite loci, or between allozyme and microsatellite locus pairs. There were no significant differences among collections in mean observed heterozygosities for either marker set. Over microsatellite loci, the site 1 collection had the greatest mean number of alleles, but this was not significant (ANOVA, $P = 0.98$) due to the high variance over loci.

The allozyme and microsatellite loci differed markedly in respect to their relative conformity to Hardy Weinberg Equilibrium (HWE). Deviations from HWE expectations were generally small for allozymes, with only a single locus out of HWE over all populations after Bonferroni correction ($\alpha = 0.05/11 = 0.0045$). Two allozyme loci were out of HWE in individual collections after Bonferroni correction: *MPI** in the site 3 collection due to heterozygote deficiency and *MDH** in the site 10 collection due to heterozygote deficiency and the site 4 collection due to heterozygote excess. There were 2 additional marginally significant ($P < 0.05$) F_{IS} values involving heterozygote excesses: *ARGK** in the site 13 collection and *GPI** in the site 5 collection. Departures from HWE over all allozyme loci were not significant in any collection after Bonferroni correction ($\alpha = 0.05/15 = 0.0033$, Table 2).

In contrast, the microsatellite loci, with few exceptions, exhibited significantly fewer heterozygotes than expected (in most cases, $P < 0.001$; $\alpha = 0.05/10 = 0.005$, Table 3). The most notable exception was Pab6, which exhibited no significant departures from HWE in any population ($P = 0.0780$, Table 3); however, departures from HWE were also not significant after Bonferroni correction for Pab9 in collections from sites 1, 8, 11, 15, 16, and 17, and, for Pab7 in the site 15 collection and Pab8 in the site 8 collection.

Among-Population Genetic Diversity

Estimated values of F_{ST} were small for both marker classes ($F_{ST} \leq 0.01$, Table 1). Across all samples assayed, the global F_{ST} values for allozymes and microsatellites was small yet significant after bootstrapping ($F_{ST} = 0.002$, $P < 0.001$; $F_{ST} = 0.001$, $P = 0.004$, respectively). Across the eight samples assayed with both marker types, the global F_{ST} value was 0.002, also significant after bootstrapping ($P < 0.001$). The allozyme locus *GPI** and the mi-

cro-satellite locus Pab4 had the greatest influence on F_{ST} , with values of 0.0018 and 0.0005, respectively, after jackknifing (Table 4). Significant genetic differentiation was detected among collections using allozymes ($P = 0.006$), microsatellites ($P < 0.0001$), and both marker classes ($P < 0.0001$) via global genic (allozyme) and genotypic (microsatellites and combined) tests (as implemented in Genepop). There was no evidence for selection at any locus: F_{ST} values for all loci plotted against heterozygosity were within the range of the expected distribution for neutral markers as determined by the Beaumont and Nichols (1996) method.

The hierarchical AMOVAs performed for both allozyme and microsatellite datasets yielded similar results, with 99.71% (allozymes) and 99.75% (microsatellites) of the variation occurring within collections. Variation among basins was nonsignificant for both marker classes (Table 5). Variation among collections was significant for allozymes ($P = < 0.0001$) but not for microsatellites ($P = 0.19062$), and F_{ST} values were significant for both allozymes ($P = < 0.0001$) and microsatellites ($P = 0.041$).

Among the eight populations analyzed with both allozymes and microsatellites, the site 15 collection (Freshwater Bay) was the most genetically divergent; it differed significantly from all common collections except sites 1 (Case Inlet) and 13 (Langley) in pairwise exact genotypic tests with at least one of the two classes of marker (Table 1). Only one of the significant differences, involving site 12 (South Hood Canal) was detected with both allozymes and microsatellites. Significant divergence in genotypic frequencies were observed in four comparisons using both marker sets, but not detected with either marker set separately after Bonferroni corrections. Three of these comparisons involved site 8 (Dyes Inlet), which differed from sites 11, 12, and 16. The fourth comparison revealed differences between sites 11 and 13, again only with both sets of markers combined.

Three other significant differences among common collections (sites 6, 8, and 11) were detected with microsatellites alone. Significant differences between site 15 and sites 3, 4, and 14 for allozymes, and site 2 for microsatellites were also observed. Among populations analyzed only with microsatellites, site 2 stood out as relatively distinct; it differed significantly from sites 8, 12, 13, 15, and 17.

For collections analyzed with both allozymes and microsatellites, pairwise F_{ST} values calculated with allozyme and microsatellite loci separately were correlated (Mantel test, $R^2 = 0.47$, $P = 0.032$), but were typically higher for allozymes (Fig. 2) Without the allozyme locus *GPI**, however, the mean $F_{ST} = 0.0017$, and the T test was no longer significant. There was no correlation between expected heterozygosity and F_{ST} at individual loci for either allozymes or microsatellites. Mantel tests revealed no significant correlations between F_{ST} and either linear or adjusted linear watercourse distance (distance matrix A and B, $P > 0.10$, 0.57 respectively; Fig. 2), not even including the site 17 (Alaska) outgroup (Fig. 3).

DISCUSSION

The results presented are among the first studies of marine bivalve population genetics involving both microsatellite and allozyme loci on the same sample set. In general, geoduck aggregation localities appear genetically homogenous within the region studied, although both marker classes were concordant in the detection of genetic differentiation of the Freshwater Bay (site 15) collection from others. The possible biologic significance of these findings is discussed later.

TABLE 4.

F-statistics and Jackknifed F-statistics for 11 allozyme and 7 microsatellite loci at 15 and 10 collections of *Pauopea abrupta*, respectively.

F statistics				Jackknifed			
Allozyme Locus	F _{IS}	F _{IT}	F _{ST}	Excluded locus	F _{IS}	F _{IT}	F _{ST}
ALAT	0.0429	0.0439	0.0011	ALAT	0.0288	0.0313	0.0025
MPI	0.0836	0.0871	0.0038	MPI	0.0182	0.0203	0.0021
PEPA	0.0226	0.0270	0.0045	PEPA	0.0302	0.0323	0.0022
PGDH	-0.0129	-0.0134	-0.0004	PGDH	0.0299	0.0323	0.0025
SOD	0.0165	0.0160	-0.0005	SOD	0.0310	0.0337	0.0028
ARGK	0.0031	0.0024	-0.0007	ARGK	0.0338	0.0366	0.0029
GPI	-0.0184	-0.0117	0.0066	GPI	0.0370	0.0387	0.0018
AAT1	0.0110	0.0113	0.0003	AAT1	0.0304	0.0329	0.0025
GAPDH	-0.0218	-0.0207	0.0010	GAPDH	0.0300	0.0324	0.0024
MDH	0.0199	0.0211	0.0012	MDH	0.0307	0.0332	0.0026
IDHP	0.0748	0.0776	0.0030	IDHP	0.0234	0.0257	0.0023
Total	0.0295	0.0318	0.0024	mean	0.0300	0.0324	0.0025
Microsatellite							
Locus							
Pab3	0.4841	0.4844	0.0005	Pab3	0.3440	0.3446	0.0010
Pab4	0.6422	0.6432	0.0029	Pab4	0.3165	0.3168	0.0005
Pab5	0.4369	0.4375	0.0010	Pab5	0.3526	0.3532	0.0009
Pab6	0.0449	0.0450	0.0001	Pab6	0.4169	0.4175	0.0010
Pab7	0.2683	0.2686	0.0005	Pab7	0.3797	0.3803	0.0010
Pab8	0.5058	0.5065	0.0013	Pab8	0.3395	0.3400	0.0008
Pab9	0.1446	0.1444	-0.0002	Pab9	0.4003	0.4010	0.0011
total	0.3643	0.3649	0.0009	mean	0.3649	0.3655	0.0009

Within-population Genetic Diversity

In marked contrast to the allozyme loci, most of the microsatellite loci were out of HWE. For allozyme loci, deficiencies of heterozygotes relative to the expectations of HWE have been frequently reported for invertebrates, and bivalves in particular (Zouros & Foltz 1984, Raymond et al. 1997). These deficiencies

have variously been characterized as resulting from null alleles (Foltz 1986), a Wahlund effect (David et al. 1997), inbreeding and genotype-dependent spawning (Rios et al. 1996) or selection (Ridgway 2001). Because the assumption of discrete generations, inherent to the Hardy-Weinberg principle, was violated by this study, that the allozyme loci overall were in HWE may indicate a degree of genetic stability across generations. Because little evi-

TABLE 5.

Hierarchical analysis of molecular variance (AMOVA: Weir, B.S. and Cockerham, C.C. 1984; Excoffier, L., Smouse, P., and Quattro, J. 1992. Weir, B.S., 1996.) using A) microsatellite data for collections of *P. abrupta* grouped into 4 basins: Straits (sites 14, 15), Main (sites 6, 8), Hood Canal (sites 11, 12) and Southern (sites 1, 2), and B) allozyme data for collections of *Pauopea abrupta* grouped into 4 basins: Straits (sites 14–16), Main (sites 6–9), Hood Canal (sites 11, 12) and Southern (sites 1, 3–5).

A

Source of variation	DF	Variance components	Percent variation	Fixation indices	P
Among basins	3	0.00086 Va	0.03	F _{CT} 0.00028	0.19257
Among collections within basins	4	0.00683 Vb	0.22	F _{SC} 0.00219	0.19062
Within collections	1520	3.10694 Vc	99.75	F _{ST} 0.00247	0.04106
Total	1527	3.11463			

B

Source of variation	DF	Variance components	Percent variation	Fixation indices	P
Among basins	3	-0.00133 Va	-0.06	F _{CT} -0.00062	0.79765
Among collections within basins	9	0.00762 Vb	0.36	F _{SC} 0.000355	<0.0001
Within collections	2581	2.13812 Vc	99.71	F _{ST} 0.00293	<0.0001
Total	2593	2.14440			

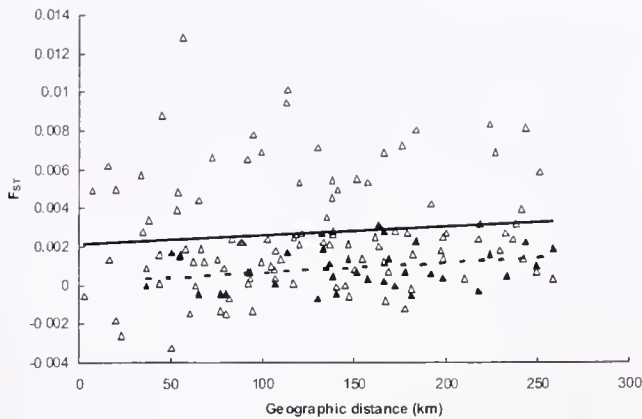


Figure 2. *Panopea abrupta* sample site pairwise genetic and geographic distances: Genetic distance, indicated as F_{ST} , over all allozyme (\square) and microsatellite (Δ) loci for each population pair, as a function of watercourse distance (km) between sites. Indicated trendlines for allozyme (solid line) and microsatellite (dashed line) loci are nonsignificant.

dence of overall heterozygote deficiency is apparent in the allozyme data, our data suggest that population level processes are unlikely to be the cause of the heterozygote deficiencies that we observed with microsatellites.

Aside from population level processes, potential causes of the observed heterozygote deficiencies in microsatellites are size homoplasy or mis-scoring of alleles, upper allele dropout, and non-amplifying alleles. Mis-scoring or inadvertent binning of neighboring alleles is akin to size homoplasy, and undoubtedly occurred to some extent in this dataset. Indeed, at a single locus, as many as

seven different microsatellite alleles with identical mobilities were detected via sequencing in the gastropod *Bulinus truncatus* (Viard et al. 1998). Given the high variability of the microsatellite loci used in this study, and the high resolution of the genotyping platform, alleles with very close mobilities are unlikely to have occurred (or to have been inadvertently binned) with a high enough frequency to produce the strong heterozygote deficiencies observed. Upper allele dropout was minimized by both the optimization of PCR conditions and the exploitation of the high sensitivity of the genotyping platform (see Materials and Methods); two alleles could differ in fluorescence intensity by more than 50,000 relative fluorescence units (RFUs), and still be accurately scored via internal lane standards. Even though observed heterozygosities were slightly lower on the less sensitive ABI 373 slab gel platform whose software allowed only a lower fluorescence intensity differential between alleles (Vadopalas, unpublished data), upper allele dropout is unlikely to account for the full magnitude of the deficit of heterozygotes. We explored the possibility of mispriming as a cause of our heterozygote deficiencies during the development of the loci (Vadopalas & Bentzen 2000) by designing primers for alternate flanking sequence. Although the number of alleles present in some individuals changed either from homozygote to heterozygote or vice versa, there was no net change in observed heterozygosities (unpublished data). We were unable to find suitably conserved flanking sequence to alleviate the occurrence of putative mispriming. Thus a likely explanation for the observed heterozygote deficit in our microsatellite data is primer site sequence variation resulting in null alleles. Hedgecock et al. (2004) gave a conservative estimate of one single nucleotide polymorphism (SNP) every 82 base pairs in the Pacific oyster *Crassostrea gigas*; this figure may be considerably higher in geoduck clams. We used the Brookfield (1996) method to calculate the expected

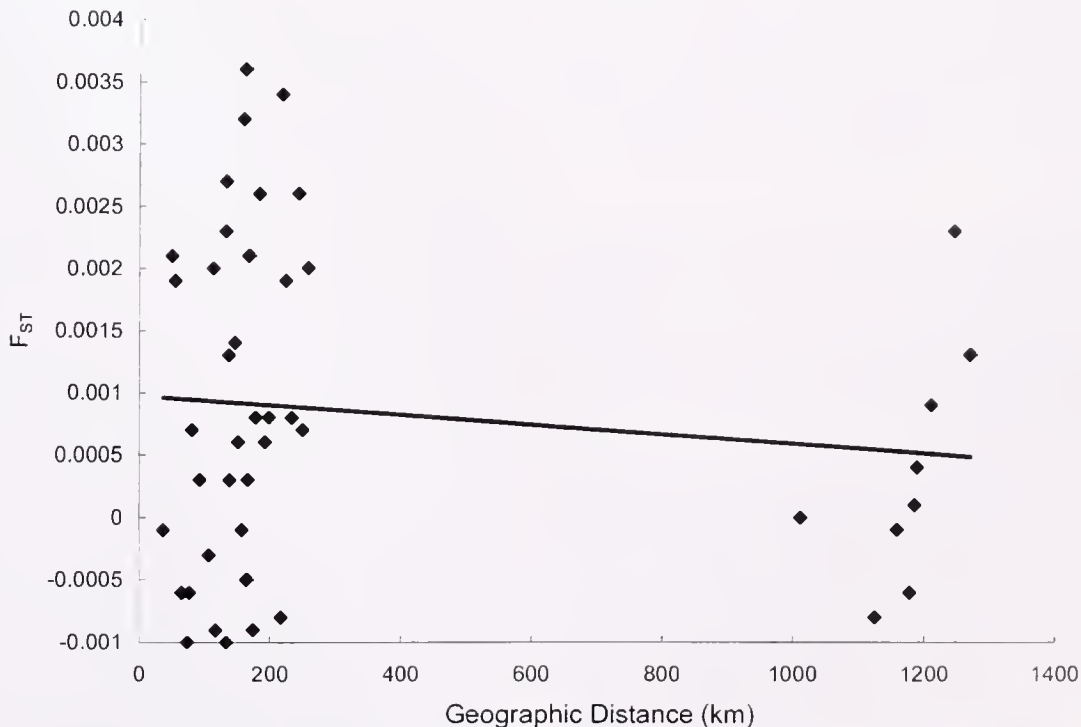


Figure 3. *Panopea abrupta* sample site pairwise genetic and geographic distances: Genetic distance, indicated as F_{ST} , over all microsatellite loci for each population pair including the SE Alaska group, as a function of watercourse distance (km) between sites. Trendline is nonsignificant.

frequency of null alleles in microsatellite loci; for all heterozygote deficient loci the expected null frequency exceeded that of the most common visible allele (Vadopalas, unpublished data). The genotypic bias recognized here is presumed to affect all populations evenly.

The mean expected heterozygosities for microsatellites (0.94) and allozymes (0.390) (Tables 2 and 3) were similar to those found in other marine bivalves. For example, the overall mean expected heterozygosity was 0.261 for 24 allozyme loci in the razor clam *Siliqua patula* (LeClair & Phelps 1994), 0.158 to 0.261 (mean 0.214) for seven allozyme loci in the greenshell mussel *Perna canaliculus* (Apte & Gardner 2001), and 0.114 to 0.696 (mean 0.413) for nine allozyme loci in the bivalve *Spisula ovalis* (David et al. 1997). For microsatellites, Huvet et al. (2000) reported a range of 0.83 to 0.96 (mean 0.88) at four loci in the Pacific oyster *Crassostrea gigas*.

Among-population Genetic Diversity

We detected similar patterns of genetic differentiation with microsatellites and allozymes. Significant differences between site 15 and all other common collections except for sites 1 and 13 were revealed by multilocus genotypic and allelic analyses using the two marker classes separately and combined (Table 1). Genetic differences between site 15 and collections within the Southern Basin were similar: no differentiation was detected between site 15 and site 1 with either marker class, whereas differences were detected between site 15 and the proximate collections from sites 3/4 and 2 with allozymes and microsatellites, respectively. The general concordance between the two marker classes in this study corroborates the findings of others (Scribner et al. 1998, Ross et al. 1999, Allendorf & Seeb 2000, De Woody & Avise 2000) and supports the notion of selective neutrality of allozymes.

Four significant pairwise exact test comparisons with all markers combined were not significant for either marker class alone. Greater power to detect differentiation may be gained by an increase in the number of markers used, rather than the number of alleles per locus or individuals per collection (Ferguson & Danzmann 1998). Although based on relatively low P values, the differentiation in three of four significant comparisons appears derived from microsatellite rather than allozyme genotype frequency variation (Table 1).

There were some discordant results between the marker classes. Differences between site 15 and sites 6, 8, and 11, detected with microsatellites, were undetected with the allozyme loci in pairwise exact tests, although the pairwise site 15-11 and 15-8 allozyme comparisons were marginally significant ($P = 0.06$ and 0.04 , respectively). Whether this possible discordance is indicative of greater statistical power attributed to markers with greater variability (Goudet et al. 1996) is unclear.

We plotted the relationship between expected heterozygosity and F_{ST} per locus over all populations, and found no significant correlation. However, pairwise F_{ST} values were generally lower for microsatellites than for allozymes (Fig. 2, Table 1), although this difference was only significant with the inclusion of the allozyme locus GPI. Jin and Chakraborty (1995) demonstrated theoretically that high mutation rates and stepwise mutation can reduce F_{ST} , a feature of microsatellites also simulated by Balloux et al. (2000). Charlesworth (1998) and Hedrick (1999) showed that compared with less variable markers, higher marker variability reduced F_{ST} values. Nevertheless, highly variable markers are powerful in

exact tests of differentiation not just by virtue of their sensitivity to gene flow (Ross et al. 1999) but also because their high variability imparts greater statistical power (Estoup et al. 1998). The paradox of lower F_{ST} parameter estimates but greater power to detect differentiation in exact tests among highly variable loci was discussed by Hauser and Ward (1998), who concluded that F_{ST} values from microsatellites can be poor estimators of genetic differentiation, and call for the use of multiple marker classes in studies of genetic structure such as used in the present study.

Biological Significance

Whether the statistically significant genetic differences among populations detected with both sets of markers together and independently are biologically significant is difficult to assess, because the pattern is inconsistent. With both allozymes and microsatellites, the differentiated pairs include site 15 and 5 other sites: 4 within Puget Sound, and the single Georgia Strait sample (16). The significant genetic differences between sites 15 and 12 and Southern Basin collections correlate with watercourse distance, whereas the differences between the outer Straits collections do not. In addition, the differences detected with microsatellites between the site 2 collection in the Southern Basin and collections in nonadjacent basins/inlets, including S.E. Alaska seem to correspond to IBD, yet with these same markers we were unable to detect differentiation between the S.E. Alaska collection and any other population surveyed. Greater differences were detected on a scale of 100 km than 1000 km; the lack of correlations between F_{ST} and distance for either class of marker (Figs. 2, 3) and the lack of differentiation between site 17 (S.E. Alaska) and all other collections in the complex except site 2 reinforce our conclusion that a simple IBD model does not apply to the current dataset.

Aside from general panmixia, there are a number of alternate explanations for our failure to find IBD. First, it may be that neither the weighted nor the unweighted distance matrix sufficiently approximates abiotic factors affecting larval dispersal, and that we missed an isolation by distance signal through an inappropriate circulation model. The complexity of Puget Sound's hydrology, bathymetry, currents, and wind-driven surface waters notwithstanding, there is a general seaward surface water flow from south to north in Puget Sound, with vertical mixing at the sills where approximately 50% of the surface outflow is cycled back to the basin of origin (Ebbesmeyer et al. 1984, Geyer & Cannon 1982). Particles released in one basin will generally be exchanged throughout the Puget Sound complex before exiting the system through the Strait of Juan de Fuca (Ebbesmeyer et al. 1988). However, water mass retention times in Hood Canal and Whidbey basins can be as high as 9 months (Cox et al. 1984) and easily exceed the 4 to 6 week geoduck pelagic stage. Thus both distance matrices oversimplify the complexities of estuarine circulation and passive larval dispersal in the Puget Sound complex.

Second, the behaviors of geoduck larvae in relation to currents, temperature, photoperiod, and phototropism are not well understood. Larval phototaxis may have a large impact on associations with a particular water mass. Because we have attempted to integrate some gross circulation rates, larval behaviors and differential rates of recycling at sills may play important roles in the degree of larval transport (Ebbesmeyer et al. 1998) and gene flow among basins that we have not sufficiently modeled.

Third, the low differentiation exhibited between the SE Alaska collection and those in Washington may be due not to high gene

flow, but instead to the tendency of highly variable microsatellites to underestimate population structure in cases of low gene flow, as demonstrated both empirically and via simulations by (Balloux et al. 2000). A combination of array size constraints (Nauta & Weissing 1996, Calabrese et al. 2001), size homoplasy/binning (Viard et al. 1998, Angers et al. 2000), and statistical noise associated with the allele:specimen ratio, perhaps strongly associated with our highly variable microsatellite loci, may confirm observations that microsatellites can underestimate genetic differentiation (Hauser & Ward 1998), especially in cases where the separation is recent.

The genetic differences found may be due to some hydrographic factors affecting geoduck genetic population structure. Differences in geoduck settlement have been hypothesized to be due to localized hydrodynamics (Zhang & Campbell 2004). The differentiation of the site 15 collection may be due to difficult emigration caused by strong oceanographic conditions that occur in the Strait of Juan de Fuca. A sill, roughly between Victoria, BC and Dungeness Spit, Washington, separates the inner and outer Strait (Fig. 1). Intense homogenization of surface and deep waters occurs at the sill, where summer water temperatures are colder than they are elsewhere in the region, perhaps serving as a barrier to successful larval immigration. A possible isolating mechanism in this general locality is suggested by the allele frequency divergence in Butter clams (*Saxidomus giganteus*) found by Johnson and Utter (1973).

The relatively few differentiated collections may reflect selection, because the differences may be primarily driven by a minority of loci linked to genes under selection. Among the allozyme loci, the significant differentiation of site 15 from other collections was driven primarily by the allozyme locus *GPI**. *GPI* seems to be under temperature selection in *Mytilus edulis* (Hall 1985) with a latitudinal gradient among alleles (Koehn et al. 1976, Koehn et al. 1984). Both temperature and salinity vary less at site 15 compared with other more estuarine localities; a similar mild selective effect might explain the observed differences in *GPI** allele frequencies in geoducks. Without *GPI**, jackknifing over collections resulted in a global F_{ST} of 0.00175 (Table 4), indicating *GPI** has the strongest effect among allozymes loci on global F_{ST} . Without site 15, jackknifing over loci resulted in an overall F_{ST} for *GPI** of 0.00088, further indication that the differentiation may be due to this locus. However, no evidence that this locus is under selection was detected using the method of Beaumont and Nichols (1996).

Another possible cause of the seemingly random genetic differentiation could be the scale on which we are sampling. Seemingly random differentiation can exist on a very fine scale due to variable settlement patterns (Larson & Julian 1999). *Panopea abrupta* larval cohorts may aggregate in clusters to increase dispersal. If larvae in the natural environment increase their drag coefficient by rafting together using a combination of byssus and mucus as we have observed in the hatchery environment (Vadopalas, unpublished data), they may remain associated until settlement. Some separation of larvae would still occur via turbulence, postsettlement byssus drifting (Sigurdsson et al. 1976), or pedal locomotion over the substrate (Cole & Beattie 1991). Nevertheless, some degree of clustered dispersal may explain our results. If it does occur, some degree of increased relatedness within, and increased differentiation among, collections on a subkilometer sampling scale may be detectable. Studies are underway to investigate microspatial genetic variation in *P. abrupta*.

We assume the geoduck collections in this study consisted of overlapping generations, because ages range from 12 to 131 for a

random sample of *P. abrupta* from Puget Sound (Goodwin & Shaul 1984). With the high longevity, early reproduction, and no apparent reproductive senility (Sloan & Robinson 1984), the mean generation time for geoduck clams is approximately 30 years; a random sample of 100 geoducks is likely to include more than 50 year classes and many overlapping generations. Because overlapping generations violate assumptions of many population genetic models (Hartl & Clark 1997), the overall effect of our random sampling of cohorts within each collection should be to minimize the effect of temporal genetic drift (Jorde & Ryman 1995). However, *P. abrupta* may use the strategy modeled by Ripley (1998) in which bivalves with high longevity and fecundity can wait many years until the relatively rare occurrence when conditions are conducive to mass reproductive success. This strategy would maintain genetic variability over long temporal scales, but may create stochastic differences on shorter temporal scales. Indeed, in another large collection for which we have age data (genotype and age data to be reported in a forthcoming study), 27% of the specimens are of a single year class. If separate year classes are genetically differentiated via sweepstakes recruitment (Hedgecock 1994) in years, intervening between episodes of strong recruitment genetic variability would be reduced within cohorts. Supporting the sweepstakes hypothesis, David et al. (1997) demonstrated significant differences between cohorts in the clam *Spisula ovalis*. Li and Hedgecock (1998) demonstrated significant genetic heterogeneity among larval *Crassostrea gigas* cohorts, and Moberg and Burton (2000) detected greater heterogeneity among recruits than among adults over similar spatial scales.

Our results are similar to those of Johnson and Black (1984) and Edmands et al. (1996) who found what appeared to be stochastic genetic differentiation in marine invertebrates (*Siphonaria jeanae* and *Strongylocentrotus purpuratus*, respectively), with genetic homogeneity on a broad spatial scale and heterogeneity on a fine scale. Similar observations for other marine taxa exist (see Shaklee & Bentzen 1998 for a review). Whether the heterogeneity detected in this study reflects temporally stable differences among local populations of geoduck clams, or are instead due to large variation in year class strength and strong bias in reproductive success among spawners is the subject of ongoing investigations.

CONCLUSION

In general, genetic homogeneity, or panmixia, was found among collections of Puget Sound geoducks, with only a few statistically significant differences that are inconsistent with an isolation by distance model. To maximize the signal of genetic differentiation relative to random noise, temporal replication is highly recommended (Waples 1998). Temporal homogeneity was assumed among our collections, but given age data currently on hand for other geoduck clam collections, such an assumption is unlikely to be accurate. A temporal Wahlund effect is possible if, for example, a large proportion of a single year class comprises the site 15 sample. Thus, it is difficult to conclude whether the genetic differences between this site and others represent long-term effects of reproductive isolation leading to separate evolutionary trajectories, thermal selection, or simply stochastic variation. Including only those collections within the Puget Sound, no significant differences were detected with allozymes, and yet four differences were detected with microsatellites. Again, this differentiation among geoduck collections may be temporally unstable over a scale much longer than a few generations. Whether such patterns

of "chaotic genetic patchiness" apply to other marine taxa in the Puget Sound region remains to be investigated. We emphasize the need to study both the spatial and temporal scales of sweepstakes recruitment, so fishery managers can incorporate genetic population structure into harvest and culture management models.

ACKNOWLEDGMENTS

The authors thank two anonymous reviewers for helpful comments and suggestions that greatly improved the manuscript. The

authors also thank and are indebted to J. Shaklee, P. O'Reilly, F. Utter, C. Friedman, L. Hauser, and J. Davis for helpful discussions; A. Bradbury, R. Sizemore, D. Rothaus, M. Ulrich, and M. Walker for providing geoduck samples; and C. Bowman, R. Colwell, A. Drape, C. Duff, B. Ingram, E. LeClair, A. Marshall, K. Obrien, K. Sweeney, and N. Switzler for help with dissections. This work was supported by Washington Sea Grant #NA76RG0119. B.V. was additionally supported by the Roy Jensen Fellowship, School of Aquatic and Fishery Sciences, University of Washington.

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SEASONAL SHELL GROWTH AND LONGEVITY IN *DONAX VARIABILIS* FROM NORTHEASTERN FLORIDA: EVIDENCE FROM OXYGEN ISOTOPES

DOUGLAS S. JONES,^{1,*} IRVY R. QUITMYER¹ AND C. FRED T. ANDRUS²

¹Florida Museum of Natural History, University of Florida, Gainesville, Florida 32611 and

²University of Georgia, Savannah River Ecology Laboratory, Aiken, South Carolina 29802

ABSTRACT The variable coquina clam, *Donax variabilis*, is one of the most common inhabitants of exposed sandy beach intertidal and shallow subtidal zones in the southeastern United States. Its exceptional burrowing and migratory behaviors are well documented, as are its biogeographic distribution and phylogenetic relationships. However, basic life history parameters such as season and rate of shell growth and longevity are poorly constrained for this species, usually estimated from size-frequency analyses of sample populations. High-resolution sampling of individual *D. variabilis* shells, and analysis of the oxygen isotopic variation in these samples, provides an alternate method of assessing shell growth and longevity in this species with a high degree of precision. Comparison of isotopic paleotemperature profiles with local seawater temperatures in the northeastern Florida study region indicates rapid shell growth (>4 mm/month) during a life span of 3 to 5 months, substantially shorter than most previous estimates. Detailed analysis of two modern shells indicates growth during spring and summer whereas four Archaic period archaeological shells revealed a summer-autumn growth record. In all cases the largest archaeological shells were substantially bigger than the largest modern shells, reflecting a greater longevity by about 2 months. Although recruitment can be fairly continuous throughout the year, size-frequency analyses of *D. variabilis* in separate years at the same locality reveal substantial interannual differences in population dynamics.

KEY WORDS: *Donax variabilis*, oxygen isotopes, shell growth, longevity, Florida

INTRODUCTION

The variable coquina clam, *Donax variabilis* Say, 1822, is one of the most common, recognizable bivalve mollusks from sandy beach habitats of the southeastern United States. It is found from Virginia to southern Florida and around the Gulf Coast to Texas (Ruppert & Fox 1988). Like other members of the Family Donacidae, *D. variabilis* is often the dominant macro-invertebrate inhabiting the high-energy environment of exposed sandy beaches where it often occurs in extremely high densities (Ansell 1983, Brown & McLachlan 1990, Pearse et al. 1942, Wilson 1999). In some cases *Donax* clams account for over 95% of the macrofaunal biomass on such beaches (McLachlan et al. 1981).

Donax clams are excellent burrowers, ideally suited to life in the surf zone (McLachlan & Young 1982, Trueman & Ansell 1969). The wedge-shaped outline of their shells facilitates penetration into the substrate. In addition to rapid burrowing, *D. variabilis* is also noted for its mobility, moving up and down the beach with the tides (Turner & Belding 1957), responding in a complex manner to wave action and especially to the acoustic shock of breaking waves (Ellers 1995a, Ellers 1995b). On longer time scales, Ruppert and Fox (1988) report a seasonal cycle of migration across the beach zone, down into the shallow sublittoral in fall, and returning onto the beach as juveniles in the late winter. This rather nomadic lifestyle, combined with its supreme ability as a rapid burrower, has enabled this species to colonize the sands of exposed beaches that otherwise are fairly devoid of macrofauna (Wilson 1999).

Despite its familiarity to scientists and shell collectors alike, the taxonomic status of *D. variabilis* has been debated for decades. Morrison (1971) envisioned 2 largely sympatric species, *D. variabilis* and *Donax parvula* Philippi, 1849, with the former further subdivided into a subspecies occurring in the southwestern portion of the range, *D. variabilis roemeri* Philippi, 1849. By contrast, Abbott (1974) and Dance (1990) considered *D. parvula* an ecomorph of *D. variabilis*. From studies of morphology, genetics and

ecology, Nelson et al. (1993) concluded that *D. variabilis* and *D. parvula* were highly similar, but distinct species.

Using molecular techniques (RAPD DNA markers), Adamkewicz and Harasewych (1996) seem to have resolved the lingering systematics issues as well as simplified our understanding of the biogeography of *Donax* in eastern North America. They found no evidence for the existence of the subspecies *D. variabilis roemeri* and found *D. parvula* to be indistinguishable from the subtidal *Donax fossor* Say, 1822, with the latter synonym having taxonomic priority. Their biogeographic assessment has *D. variabilis* sharing both the Atlantic and Gulf coasts with a smaller, subtidal species, *D. fossor* on the Atlantic coast and *Donax texianus* Philippi, 1847, in the Gulf of Mexico. The Florida peninsula separates the two subtidal species (Adamkewicz & Harasewych 1996).

The principal distinguishing feature between *D. variabilis* and *D. fossor*/*D. parvula* is the angle of the dorsal margins of the shell on either side of the umbo (Wilson 1999). Unfortunately, juvenile specimens often cannot be assigned to one species or the other (e.g., Bonsdorff & Nelson 1992) and ecophenotypic variation can further complicate specific determination, particularly in the field. Hence, we considered all small shells (≤ 3 mm) collected for this study to be *D. variabilis*, combining them with the larger specimens with which they were collected.

Despite its ecological importance, abundance and visibility on exposed sandy beaches, several key life history parameters of *D. variabilis* such as growth rate and longevity are known only to a first approximation with widely divergent estimates in the literature. This is particularly significant when *D. variabilis* is used as a model organism in ecological studies involving population dynamics and energy budgets on sandy beaches (Wilson 1999), or in investigations of the roles of biotic versus abiotic factors in determining spatial distribution and recruitment of beach fauna (Schoeman & Richardson 2002). Longevity estimates for *D. variabilis* range from less than 1 year (e.g., Bonsdorff & Nelson 1992) to higher than 3 years (e.g., Morrison 1971). At least part of the explanation for the lack of accurate data is the traditional method for assessing growth and longevity in this species—analysis of

*Corresponding author. E-mail: dsjones@flmnh.ufl.edu

size-frequency diagrams—an approach with many inherent problems (Mikkelsen 1985). Other techniques such as mark-and-recapture studies are impractical because of the movements and high mortality of these clams (Mikkelsen 1985), and growth rings on the shell are indistinct, not useful for age determination (Wilson 1999). In this study we use oxygen isotopic variation in shell carbonate to produce high-resolution records of seasonal shell growth and longevity in *D. variabilis*.

Florida coquina clams normally are not exploited as a food resource by humans today; however, archaeological sites in northeastern Florida attest to the fact that preColumbian people consumed vast quantities of coquina clams between the middle Archaic (ca. 5700 y BP) and St. Johns (ca. 400 y BP) periods (Milanich 1994). This study of growth and longevity in *D. variabilis* originated from an investigation of archaeological specimens recovered from coastal shell middens in northeastern Florida to determine if there was a seasonality to ancient shellfish harvest (Jones et al. 2003, Quitmyer et al. 2004). In addition to examining archaeological shells, year-round collections of living coquina clams were made to establish a modern analogue for comparative purposes. Oxygen isotopic profiles of both modern and archaeological shells document rapid seasonal growth and shorter life spans than previously acknowledged for this diminutive but important bivalve species.

MATERIALS AND METHODS

The earliest evidence for human use of marine resources in northeastern Florida comes from the many Archaic period coastal archaeological sites recognized from this region (Milanich 1994). Particularly abundant in these shell middens are specimens of *D. variabilis* that often dominate the zoo archaeological component of any particular site. Collections of *D. variabilis* have been made at many of the more prominent excavation sites by archaeologists and students from the University of Florida (Quitmyer et al. 2004). These are housed in the collections of the Environmental Archaeology Program at the Florida Museum of Natural History, University of Florida, Gainesville.

Four archaeological shells of *D. variabilis* were selected for oxygen isotopic analysis from among the largest specimens in the museum collections. Large specimens were chosen to increase the likelihood of measuring maximum longevity. Two well-preserved shells from the Preceramic Archaic were selected, one each from the Crescent Beach (4240 ± 80 corr. ^{14}C yr BP) and Spencer's Midden (5570 ± 80 corr. ^{14}C yr BP) sites (Fig. 1). The shell lengths of these specimens were 23.1 mm and 21.5 mm, respectively. In addition, two well-preserved specimens were selected from the Orange Period Archaic material, one shell (21.3 mm) from the Rollins Site (3760 ± 60 corr. ^{14}C yr BP) and another (20.5 mm) from the Guana Shell Ring (3600 ± 50 corr. ^{14}C yr BP).

To establish a modern analogue for comparison with the archaeological coquina shell material, *D. variabilis* specimens were live-collected at monthly intervals at Matanzas Beach between December 2001 to November 2002. The collection site on Anastasia Island is located just south of the beach access ramp from Fort Matanzas National Monument (N 29.7174° ; W 81.2310°), north of the Matanzas Inlet, in the same vicinity and water depths where indigenous people could have harvested coquina clams thousands of years ago (see Fig. 1). Specimens were collected in the intertidal and shallow subtidal zones (≤ 1.5 -m depth) for a consistent 40-min interval each month. Live clams were water-

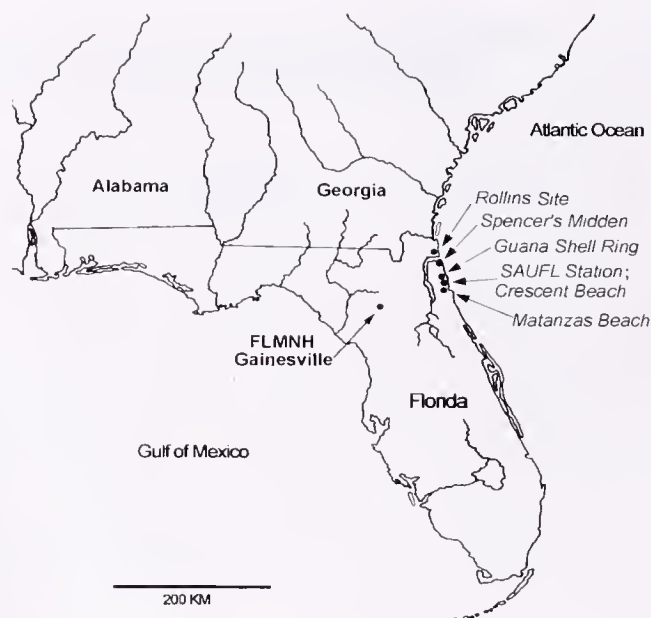


Figure 1. Location map showing archaeological midden sites along the northeastern coast of Florida, including site of modern *D. variabilis* collection at Matanzas Beach and SAUFL station at Crescent Beach.

sieved from bulk samples scooped from the sandy bottom using a 1.59 mm-gauge brass sediment sieve, identical to that used to sieve archaeological materials. The clams were bagged, stored in a cooler, and transported back to the museum where they were quickly frozen and stored. Soft tissues were removed by water maceration at a later date.

A comparable assemblage of *D. variabilis* shells, live-collected at monthly intervals between January to December 1988 at the same locality, was also available at the museum. These shells were collected by Dr. Michael Russo during the course of research in the Department of Anthropology at the University of Florida (Quitmyer et al. 2004).

The maximum shell length, anterior to posterior, was measured for each shell in every monthly collection from both data sets using a dial caliper. The monthly shell length frequencies were calculated and plotted in 1-mm increments to facilitate the temporal comparison of shell size variation in the populations. Similar size-frequency data were constructed by Quitmyer et al. (2004) for archaeological coquina clam shells excavated from middens in northeastern Florida. Two of the largest modern shells were selected for stable isotopic analysis (13.1 mm, 13.2 mm). These specimens were collected by Russo in July 1988.

When carefully sampled in ontogenetic sequence from the umbo to the ventral shell margin (Fig. 2), the pattern of oxygen isotopic variation across a bivalve shell can provide a wealth of information about seasonal growth, shell growth rates, longevity, and season of death (Jones & Quitmyer 1996, Jones et al. 1983, Wefer & Berger 1991). To assess these parameters in *D. variabilis*, and to look at possible changes through time, sequential samples of shell carbonate were recovered from the two modern and four archaeological shells.

Prior to sampling each valve was cleaned following Andrus and Crowe (2002), with a wire brush under distilled water and then treated with a 30% solution of H_2O_2 . After rinsing in distilled water and drying in a vacuum oven, the samples were mounted



Figure 2. Archaeological *D. variabilis* shell from Guana Shell Ring site showing sampling grooves from milling procedure to collect powders for oxygen isotopic analyses. Scale bar divisions = 2 mm.

onto glass slides with epoxy and fixed to the sample stage of a Merchantek EO Micromill at the Savannah River Ecology Laboratory. Carbonate samples were milled from the outer surface of the valve in shallow, 25 μm -deep grooves parallel to the growth lines. Each sampling groove was approximately 100- μm wide. The spacing between adjacent grooves averaged 200 μm with grooves near the ventral margin slightly more closely spaced than those near the umbo, reflecting the slowing of shell growth with age (see Fig. 2).

The isotopic analyses were conducted in the Light Stable Isotope Mass Spectrometry Laboratory, Department of Geological Sciences, University of Florida. The powdered CaCO_3 samples were analyzed according to standard techniques (Jones & Quitmyer 1996) which involved an initial reaction *in vacuo* with 100% orthophosphoric acid at 90°C for 0.25 h. An on-line automated carbonate-preparation system facilitated the production and purification of the evolved CO_2 gas. The isotopic differences between the derived CO_2 gas and the VPDB standard were determined with a VG Isogas PRISM Series I mass spectrometer equipped with triple collectors and micro-inlet system. All values are reported in standard δ notation where:

$$\delta^{18}\text{O} = [({}^{18}\text{O}/{}^{16}\text{O})_{\text{sample}} / ({}^{18}\text{O}/{}^{16}\text{O})_{\text{standard}} - 1] \times 10^3 \text{ per mil (‰)}$$

The weight of the individual microsamples was so low that replicates of unknowns could not be run and often required that adjacent samples be combined. However, replicated standards run before and after sample strings varied by less than $\pm 0.1\text{‰}$.

The $\delta^{18}\text{O}$ value of seawater from the Matanzas Beach collection site was measured via CO_2 equilibration following Socki et al. (1992) at the University of Georgia, Geology Stable Isotope Laboratory. Five monthly samples were analyzed (December to March & August), providing a measure of seasonal $\delta^{18}\text{O}$ range. Precision was estimated based on analysis of the laboratory working standard of Athens tap water to be ± 0.05 (1 σ). The $\delta^{18}\text{O}$ values of seawater at the Matanzas Beach collection site were very consistent (Dec. 1.0‰; Jan. 1.2‰; Feb. 1.0‰; Mar. 1.1‰; Aug. 1.1‰), averaging 1.08‰ SMOW ($\pm 0.08\text{‰}$, 1 σ ; maximum range = 0.2‰).

We calculated the temperature of the water in which the shell carbonate formed using the paleotemperature equation of Gross-

man and Ku (1986) for the temperature-dependent fractionation of aragonite in mollusks relative to seawater:

$$T(^{\circ}\text{C}) = 21.8 - 4.69 [\text{shell } \delta^{18}\text{O}_{\text{VPDB}} - (\text{seawater } \delta^{18}\text{O}_{\text{SMOW}} - 0.2\text{‰})].$$

The paleotemperature data from the coquina shells are presented in ontogenetic sequence and graphed to the mean weekly seawater temperature curve based on historical measurements made in 1988 at the nearby St. Augustine National Data Buoy Center station, SAUFL (<http://www.ndbc.noaa.gov/Maps/Florida.shtml>).

RESULTS

At Matanzas Beach, 2,493 specimens of *D. variabilis* were collected in 1988 by Russo and measured as part of this study. Another 2,545 specimens were measured from the collection made in 2001 to 2002. This latter number represents the total of all specimens collected each month except April 2002 when 28,372 specimens were collected during the usual 40-min regimen. A subset of shells (344 specimens) equal in weight to the previous month's sample (March) was selected from the April 2002 sample for measurement. The monthly length-frequency relationships for each of these samples are illustrated in Figures 3 and 4.

Each of the monthly graphs for 1988 (see Fig. 3) reveals a concentration of specimens at the smaller sizes. During winter (December to February) most of the shells in the population ranged between 2–6 mm, reflecting a preponderance of new recruits entering the population. A small percentage of larger shells were observed, up to 12 mm. In the spring (March to May) clams were more abundant than during any other season. Most shell lengths ranged between 2–10 mm with peaks at 3, 5, and 7 mm, indicative of shell growth and continued recruitment. A few specimens in the May sample reached 14 mm. The summer quarter (June to August) was marked by a very strong peak at 3 mm indicating that recruitment remained vigorous. Shell lengths ranged between 2–13 mm. Although individuals were scarce compared with other seasons, the greatest proportion of large shells was encountered in summer (see Fig. 3). By autumn (September to November) the average shell length of the population had declined with a major influx of juvenile shells in the 2–5 mm range and a decrease in the percentage of large shells.

The monthly graphs for 2001 to 2002 (see Fig. 4) show similarities and significant differences from those of 1988. The winter months were characterized by specimens in the range of 1–11 mm. Peaks at 2, 4, and 5 mm in successive months reflect an influx of new recruits in December followed by individual shell growth and a concomitant increase in the average shell length throughout the winter. The continuation of this pattern during spring resulted in peaks at 3, 7, and 10 mm. Except for June 2002, the sample sizes in July and August were very small, insufficient for a robust interpretation of patterns. The autumn samples show a very strong peak at 3 mm and also some of the largest shells recovered in either year of collecting, up to 15 mm. Heavy recruitment is indicated as well as individual shell growth by older specimens.

High-resolution milling of the *D. variabilis* shells resulted in powdered CaCO_3 samples whose weights were toward the lower limits of those typically analyzed. Therefore, successive samples were combined to insure a sufficient sample size. With respect to the modern shells, this resulted in 8 unique samples for specimen

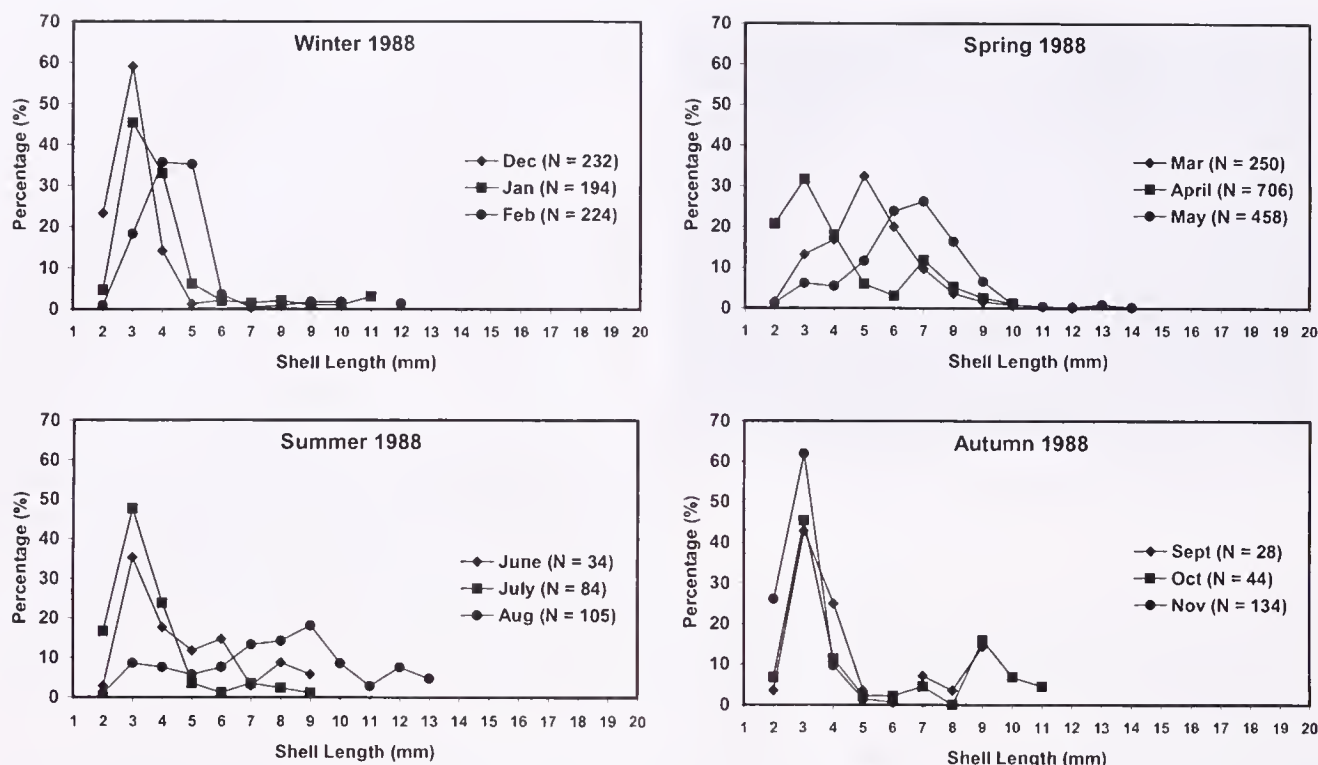


Figure 3. Shell length-frequency diagrams for *D. variabilis* collected at monthly intervals during 1988 from Matanzas Beach, FL.

#1 (13.1 mm) and 13 unique samples for specimen #2 (13.2 mm). Oxygen isotopic analyses of these samples produced values that ranged from 1.91‰ to 0.03‰ for specimen #1 and from 1.61‰ to 0.83‰ for specimen #2.

Figure 5 shows the mean weekly sea surface temperature variation at Matanzas Beach for 1988 based on historical measurements made at the nearby St. Augustine National Data Center Buoy station, SAUFL. The oxygen isotopic data from two shells col-

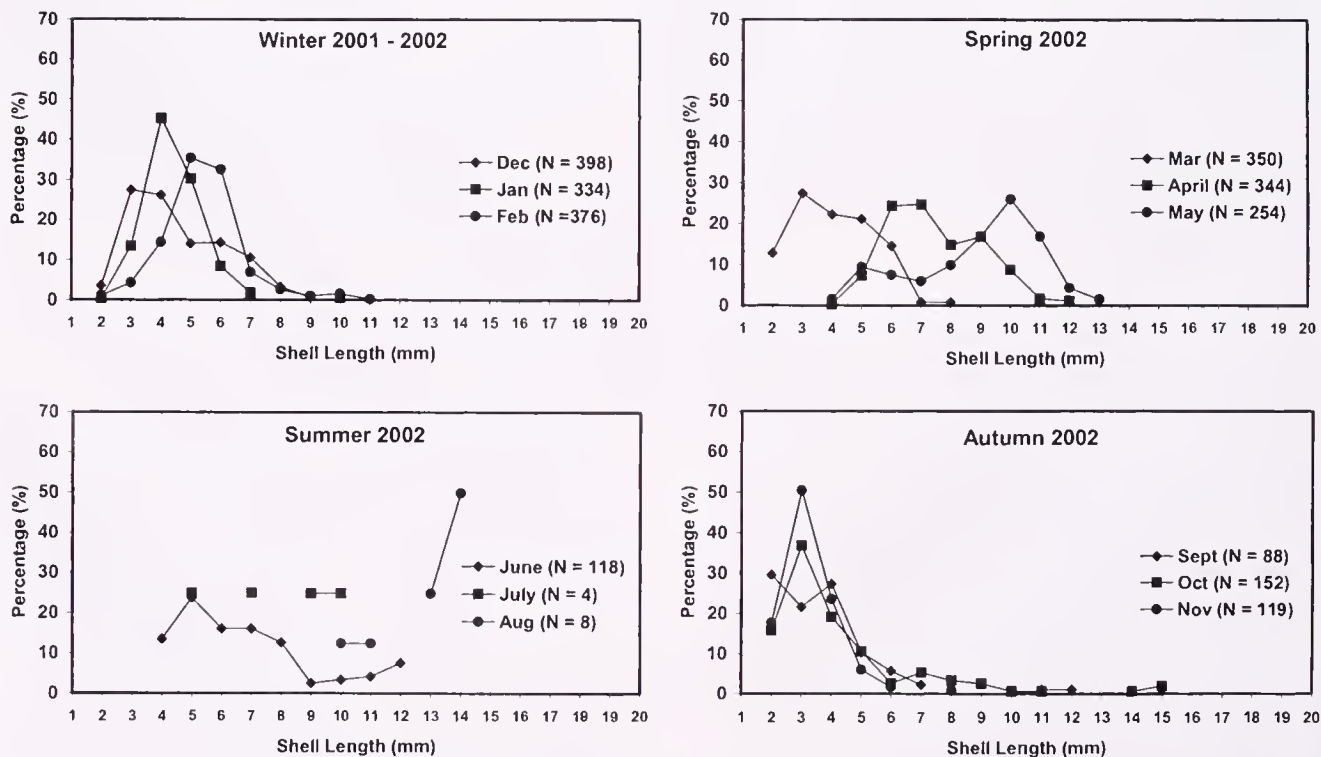


Figure 4. Shell length-frequency diagrams for *D. variabilis* collected at monthly intervals from December 2001 to November 2002 at Matanzas Beach, FL.

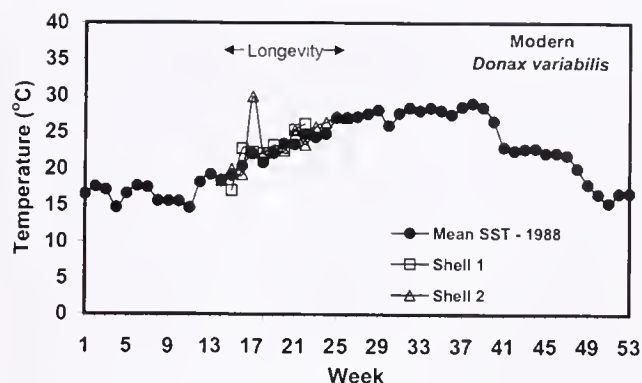


Figure 5. Weekly mean sea surface temperature data (black circles) from the SAUFL station for 1988 with isotopic paleotemperature profiles from two modern specimens of *D. variabilis* plotted in open symbols. Longevity of specimens corresponds to the length of the isotopic records, approximately 13 wk (14–26).

lected in July 1988 were converted to paleotemperatures and overlain in ontogenetic sequence onto the portion of the temperature curve that provided the best fit.

With the exception of only one data point, the isotopic paleotemperature data exhibit an excellent fit to the segment of the curve corresponding to spring and early summer. The exceptionally close match confirms the absence of vital effects influencing shell $\delta^{18}\text{O}$ values in *D. variabilis*, as in most mollusks (Wefer & Berger 1991). The shell-edge paleotemperature values correspond nicely with the water temperature at the time of collection in early July 1988. The isotopic records indicate that shell growth in both specimens occurred over a 13-week interval, approximately corresponding to weeks 14 to 26 of the year (mid April to early July).

In a similar manner, the four larger archaeological shells were sampled and analyzed for oxygen isotopic ratios. A total of 18 samples was analyzed from the Crescent Beach specimen. The $\delta^{18}\text{O}$ values ranged from 0.29‰ to 1.55‰. From the Spencer's Midden shell a total of 20 samples was analyzed whose $\delta^{18}\text{O}$ values ranged between 1.16‰ and 1.37‰. A total of 20 samples from the Rollins Site shell yielded $\delta^{18}\text{O}$ values that ranged between 1.26‰ and 1.96‰. From the Guana Shell Ring specimen, 13 samples were analyzed. These produced $\delta^{18}\text{O}$ values that ranged from 0.88‰ to 1.49‰.

The oxygen isotopic data were converted to paleotemperatures and these were overlain onto the seawater temperature curve (Fig. 6). The results are comparable with those achieved for the modern shells with a few important differences. The paleotemperature profiles for the two Preceramic Archaic shells from the Crescent Beach and Spencer's Midden sites show the closest correspondence with weeks 25 to 45 of the annual temperature curve. This pattern suggests shell growth occurred for about 21 weeks, from late June into mid-November. The paleotemperature profiles for the two younger shells from the Orange Period Archaic sites (Rollins and Guana Shell Ring) reveal a similar pattern. Shell growth over a period of about 20 weeks is indicated, corresponding to weeks 24 to 43 of the year, mid-June to late October.

All four archaeological shells were larger in size than any of the modern shells collected as part of this study. The results indicate that the ancient clams achieved their larger size by growing for a longer period of time (i.e., 8–9 wk longer) than their modern counterparts. Whereas maximum longevity among the modern shells was approximately 3 months, the archaeological specimens

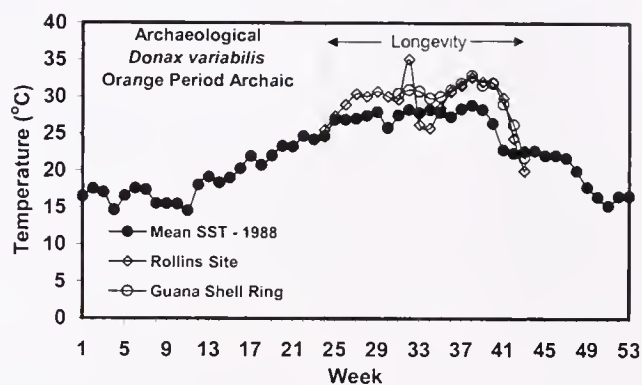
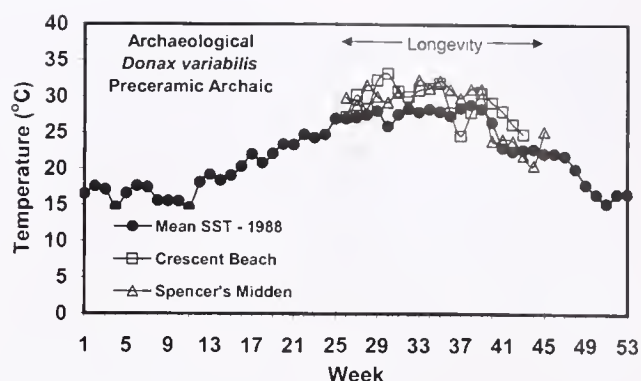


Figure 6. Weekly mean sea surface temperature data (black circles) from the SAUFL station for 1988 with isotopic paleotemperature profiles from two Preceramic Archaic shells (upper) and two Orange Period Archaic shells (lower) of *D. variabilis* plotted in open symbols. Longevity estimates for ancient shells correspond to the lengths of the isotopic records, approximately 21 wk (25–45, upper) and 20 wk (24–43, lower).

lived for up to 5 months. The paleotemperature records indicate that each of the four archaeological shells was harvested in autumn. Although the seasonal pattern of paleotemperature change recorded by the oxygen isotopes in the archaeological shells mirrors that of modern seawater at this site, the values reveal a consistent negative offset from modern conditions. The paleotemperature values from both archaeological periods reflect ocean water conditions in this region that were warmer than modern temperatures by about 3.5°C.

DISCUSSION

Despite its abundance and familiarity to scientists and shell collectors, key life history parameters such as growth rate and longevity are poorly constrained for *D. variabilis*. As this short-lived but abundant bivalve finds increasing use in ecological modeling of exposed beach ecosystems, it is essential that basic life history parameters are determined more precisely.

The oxygen isotopic evidence from modern and archaeological shells of *D. variabilis* provides the most accurate assessment of seasonal shell growth and longevity in this species to date. These data serve to refine previous estimates that were based largely on size-frequency analyses that required critical assumptions to be made about the seasonal timing of recruitment and migrations, assumptions that may or may not be warranted. Inherent problems in the interpretation of life-history information from size-

frequency data are acknowledged by several authors including Mikkelsen (1985, p. 310) who states, "... repeated sampling of the population to construct length-frequency graphs was used, although problems exist with its use."

In his overview of the biology of the genus *Donax*, Ansell (1983) reported that most species are short-lived with life spans of 1–2 y, rapid growth to maximum size, and early maturity. Morrison (1971) suggested that *D. variabilis* has a 2-year life span, and that in some cases individuals may survive a third year. Ansell (1983) reported a 1- to 2-year life cycle for *D. variabilis*. Mikkelsen (1985) estimated that *D. variabilis* in Florida grew at a rate of 3.0–3.7 mm/month in the summer months and that, "the majority of individuals probably live for approximately 1 year, with a few entering a second year" (Mikkelsen 1985, p. 310). This conclusion generally agrees with Loesch (1957) and Pearse et al. (1942) who also used length-frequency graphs to examine growth and longevity in this species.

"Bonsdorff and Nelson (1992) estimated a maximum growth rate of 3.43 mm/month for a Florida population of *D. variabilis*, at which rate the animals would grow to maturity in just 2 months" (Wilson 1999, p. 78). The oxygen isotope results achieved in this study support such rapid growth rate estimates and reduced longevity (i.e., months, not years). In fact, the growth rates calculated from our isotopic data are even higher, 4.3–4.4 mm/month for the modern specimens and 4.1–4.6 mm/month for the archaeological shells.

The isotopic data also call into question whether any of these clams actually survive for 1 full year as the modern and archaeological shell isotope records indicated life spans of 3 to 5 months, respectively. Admittedly both the modern and archaeological specimens were live-collected by humans and therefore it could be argued that they still had the potential to grow larger and live longer. However, both sets of shells were among the largest specimens out of the thousands recovered in either the modern or zooarchaeological collections, thereby minimizing this possibility. Our results cast serious doubt on previous longevity estimates for this species that hypothesize growth and survival into a second or even a third year of life.

In a study involving *D. variabilis* from the South Carolina coast, Wilson (1999, p. 69) states: "Survival of *Donax* beyond a shell length of 10–11 mm, which length it can attain in less than 1 year, was poor and these large individuals only rarely contributed more than 1% to 2% of the population." He goes on to lament that growth rings on the shell were indistinct and it was not possible to verify the age of specimens by this method. Furthermore, constant movement of beach sands and contained clams, as well as the migratory ability of *Donax variabilis*, prevented the use of a "mark-and-recapture" technique for growth measurement. These same observations hold true regarding the populations in north-eastern Florida and reinforce the value of the oxygen isotopic technique for growth rate and age determination.

The length-frequency graphs (see Figs. 3, 4) from the monthly collections of 1988 and 2001 to 2002 also reinforce Wilson's observation concerning the rarity of large individuals. The maximum SL in the modern Florida samples was 15 mm and such large shells were very rare. In contrast, the *D. variabilis* shells recovered from the archaeological shell midden sites ranged up to 25 mm and specimens in the 10–22 mm range were most common (Quitmyer et al. 2004). It seems clear from the zooarchaeological analyses that ancient harvesting strategies involved selection for larger shell

sizes, but it is not clear why the coquina shells from the ancient past exceed the size of modern shells from the same vicinity.

The presence of small clams in the population virtually year-round suggests that recruitment to the population may take place throughout the year. This was especially noticeable in the 1988 samples (see Fig. 3) and has been mentioned as a possibility by previous authors (e.g., Bonsdorff & Nelson 1992). The pattern in 2001 to 2002 (see Fig. 4) was somewhat different with continuous recruitment indicated during September to March and weak or negligible recruitment over the spring and summer months. Leber (1982) recorded juvenile recruitment to a North Carolina population occurring in February and November. The February settlement was indicative of a winter spawning. At the lower latitude of Florida, it seems reasonable that such winter spawnings could occur with greater regularity. The differences in the monthly shell size distributions between the 2 years sampled here suggest considerable year-to-year variability in the population dynamics at this northern Florida locality.

The population structure observed by Wilson (1999) at Wailes Island, South Carolina, suggested an annual cycle of settlement and growth, with reproduction and death in spring, although questions of a second, lesser spawning in autumn remain. A similar pattern was evident in the Florida population of *D. variabilis* discussed by Bonsdorff and Nelson (1992). Two spawnings per year have been reported for *D. sordidus* (McLachlan & van der Horst 1979) and for *D. semistratus* (Neuberger-Cywiak et al. 1990), although a single spawning per year is the default condition reported or assumed for most other species of *Donax*, even the short-lived, tropical species. However, the evidence is somewhat equivocal as Ansell et al. (1972) suggest that individuals of species like *D. incarnatus* may be in spawning condition at all times. Wilson (1999) acknowledges that the same might be said for the Wailes Island *D. variabilis* population, although he believed the majority of individuals were semelparous.

The size-frequency data collected as part of this study support the emerging realization that spawning and recruitment can vary considerably from 1 year to the next in *D. variabilis* and need not conform to the pattern of one major seasonal spawning per annum. Such an interpretation agrees with Ansell's (1983) observation that the typical spawning pattern for *Donax* consists of repeated spawnings by individuals over an extended spawning season. Following maturity, Ansell (1983) reports that reproduction is potentially continuous with repeated partial spawnings, but growth, reproduction, and settlement may all show seasonal responses to environmental change. Certainly our data from 1988 argue against the statement by Sastre (1984) that *Donax denticulatus* from Puerto Rico is the only known species of *Donax* with continuous recruitment.

Whereas the size-frequency analyses of the monthly samples provide important insights into the population dynamics of *D. variabilis* along the northeastern Florida coast, there are better methods available to investigate specific life history parameters such as age and growth rate. One such technique is the high-resolution sampling and analysis of oxygen isotopic variation in shell carbonate used here. This approach has yielded the most detailed records of seasonal shell growth and longevity available to date for this species. It is also applicable to archaeological and fossil shells. The excellent correspondence between paleotemperatures derived from oxygen isotopes and the measured seasonal variation in water temperature at the site made it possible to reconstruct the timing and duration of shell growth to a weekly level

of resolution. Life spans that proved to be appreciably shorter than generally acknowledged in the literature, 3 to 5 months in duration, represent an unanticipated outcome of this study. Whereas budget constraints limited the number of shells analyzed isotopically in this study, it would be highly desirable to expand the size and geographic breadth of the sampling to broaden the scope of this investigation.

Several important differences were observed between the modern and archaeological *D. variabilis* shells. These are intriguing and warrant additional study as well. The questions arising from these observations can be grouped into 3 broad categories: archaeological, biologic, and climatic. With respect to the first, all four archaeological shell records, from distinct Archaic Period time horizons, indicated shellfish harvest in the autumn. This suggests a clear seasonal pattern of behavior by indigenous peoples that needs to be verified through the analysis of additional shells from other sites and time periods (Quitmyer et al. 2004).

The biologic differences include greater maximum size and longevity for the archaeological shells, an extended growing season, and a shift in the growing season from spring-early summer to mid summer-autumn. Isotopic analyses of additional modern and archaeological shells will be necessary to document the extent of these differences and gauge their changes through time. The possibility of size reduction from over-fishing, observed in many ma-

rine species (Jackson et al. 2001) and in other bivalves such as *Mercenaria* spp. from Florida midden sites (Quitmyer & Jones 2000), must be considered. Finally, the warmer water temperatures recorded by the archaeological shell isotopes (ca. 3.5 °C) may reflect the midHolocene climatic maximum in this region (Jones et al. 2003). By attempting additional oxygen isotopic analyses of *D. variabilis* shells from older as well as younger archaeological time periods, it should be possible to chart the history of water temperature change in this region since the last glacial episode.

ACKNOWLEDGMENTS

The authors thank Dr. Michael Russo, National Park Service, Southeastern Center, Tallahassee, for sharing unpublished data in the support of this research. Jenna Chojnowsk, Catherine Liu, Nick Moskowitz, Kate Quitmyer, Erin Thornton, and Brian Worthington assisted with fieldwork, sample preparation, data acquisition, and curation. We acknowledge the Invertebrate Paleontology and Environmental Archaeology Laboratories, Florida Museum of Natural History, for supplying essential resources. US Department of Energy grant #DE-FC09-96SR18546 (CFTA) provided partial support of this research. We appreciate the analytical assistance given by Dr. Jason Curtis of the Light Stable Isotope Mass Spectrometry Laboratory, Department of Geological Sciences, University of Florida.

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THE EFFECT OF TEMPERATURE ON CLEARANCE RATE AND OXYGEN CONSUMPTION OF SCALLOPS, *CHLAMYS FARRERI*

ZHANG JIHONG,¹ J. G. FANG,^{1*} A. J. S. HAWKINS² AND P. L. PASCOE²

¹Yellow Sea Fisheries Research Institute, 106 Nanjing Road, 266071 Qingdao, People's Republic of China; ²Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth, PL1 3DH, United Kingdom

ABSTRACT Rates of particle clearance and oxygen consumption were measured over full natural ranges of seawater temperature (1 °C to 30 °C) experienced by the scallop *Chlamys farreri* cultured in Shandong Province, People's Republic of China, comparing both acclimated and non-acclimated responses. Particle clearance rate varied in hyperbolic relation with acclimated temperature of maximal values of 10 to 15 L h⁻¹ g⁻¹ dry wt, occurring in acclimated scallops between 20 °C and 25 °C, with significant reductions at temperatures above about 26 °C, particularly in non-acclimated scallops ($P < 0.001$). Oxygen consumption rate increased throughout the range of temperature. Based in part on responses defined here, a dynamic model of physiology and growth predicts that net energy balance becomes negative with short-term adjustments in seawater temperature from 15 °C to above 26 °C, when soft tissue wasting may be as much as 3.5% per day at 30 °C. These predictions are consistent with the loss of condition reported by farmers under such conditions, and help to establish that similar naturally occurring transient increases in temperature have at least in part been responsible for high coincident mortalities observed in cultured *C. farreri* during recent years.

KEY WORDS: scallop, *Chlamys farreri*, clearance rate, oxygen consumption rate, temperature, net energy balance, mortality

INTRODUCTION

Chlamys farreri is one of the main species of shellfish cultivated in China (Guo et al. 1999). However, since the 1990s, a sharp increase in mortality has compromised production, and seems linked with both abrupt temperature fluctuations and high temperature maxima in recent years (Wang & Yang 1994, Shao et al. 1996, Li & Mou 1999, Zhang & Yang 1999). The effects of environmental variables on physiologic components of growth have often been studied in bivalve molluscs (Bayne 1976, Hawkins & Bayne 1992, Albentosa et al. 1994, Espina & Buckle-Ramirez 1994, Navarro & Iglesias 1995, Urrutia et al. 1996). Whereas this includes work on scallops, effects of temperature change are poorly documented in *C. farreri* and other species (Wang et al. 1999, Yuan et al. 2000).

In China, as in several other regions, the combination of a rapidly developing mariculture industry and the increasing impact of human activities on the coastal environment emphasizes the need for predicting and monitoring the carrying capacity for shellfish culture. Carrying capacity assessment, particularly in areas practicing polyculture, is a complex issue that needs to address the interaction of many variables throughout the year or growing period. Fisheries managers are increasingly looking to models of physiologic processes and growth in cultured species (e.g., Pouvreau et al. 2000, Solidoro et al. 2000, Ren & Ross 2001, Hawkins et al. 2002), for integration within ecosystem models (e.g., Nunes et al. 2003, Bacher et al. in press; Duarte et al. in press), to help them form strategies for production and development.

In this study, we describe thermal responses in rates of particle clearance and oxygen consumption in both acclimated and nonac-

climated *C. farreri*. These responses have been used in the associated development of a dynamic model of responsive feeding, metabolism, and growth in *C. farreri* cultured in Sanggou Bay (Hawkins et al. 2002). Here, to better understand the high mortalities observed during recent years (refer to earlier), we also used that model to simulate net energy balance under a range of culture scenarios, which include abrupt changes in temperature within the range of those known to occur naturally.

MATERIALS AND METHODS

Animal Collection, Maintenance, and Treatment

Cultured *Chlamys farreri* were collected from lantern nets suspended within Sanggou Bay, Shandong Province, P.R. China (37.1°N, 122.5°E). Scallops were maintained in a 20 L aquarium in which 10 L of water were changed and about 1×10^9 cells of *Phaeodactylum tricornutum* were added daily. The size, weight, and treatment of scallops are summarized in Table 1. In this study, 60 scallops were used in experiments on clearance rate, and 30 for determination of oxygen consumption rate.

Acclimation and Temperature Control

In the acclimation experiments the temperature of the water was adjusted by 1 °C each day to reach the desired temperature and kept at each experimental temperature for 3 d before measuring clearance rates. Temperature changes were therefore gradual, to better approximate nature, when we assumed a final (standardized) period of 3 d acclimation, which we acknowledge may not have been complete. In nonacclimated comparisons, changes in temperature were made by decreasing or increasing from 15 °C directly to the other temperatures, as detailed in Table 1. Additionally, animals were also subjected to an instantaneous decrease from 30 °C to 23 °C, and in this case measurements were taken after 24 h and 96 h.

Physiological Measurements

Clearance Rate

Individual clearance rates (CR; liters of water cleared of particles $>2 \mu\text{m}$ diameter h⁻¹) were measured using a static system.

This work was funded in part by a European Community INCO-DC project ERBIC18CT980291 entitled "Carrying capacity and impact of aquaculture on the environment in Chinese bays," a Core Strategic Research Project entitled "Scaling Biodiversity, and the consequences of change" of the Plymouth Marine Laboratory, and The Chinese National Science Foundation project (no. 30271021) entitled "Study on Interaction between Intensive Bivalves culture and Environment in Coastal waters" and National Key Basic Research Program (G1999012012).

*Corresponding author. E-mail: Fangjg@ysfri.ac.cn

TABLE 1.

Sizes, weights and experimental treatments for measures of clearance rate in *Chlamys farreri*.

Date of Scallop Collection	Treatment/Temperature (°C)	Mean Shell Length (mm) (±SD)	Tissue Dry Weight (g) (±SD)
February, 2000	Acclimated at 1, 3, 5, 10, 15	54.18 ± 4.04	1.182 ± 0.271
February, 2000	Non-acclimated: 15 to 8, 20, 23, 26, 27.5, 30 and from 30 to 23 (measured after 24 and 96 h)	55.22 ± 3.91	0.773 ± 0.196
June, 2000	Acclimated at 23, 25, 27, 28, 29, 30	40.46 ± 9.49	0.145 ± 0.088

Ten to 15 individuals were individually placed in separate beakers each containing 3 L of filtered seawater at a constant temperature. An additional 3-L beaker without a scallop was used as a control. The water was mixed by gentle aeration to avoid any physical disturbance. After a period of 15 min, to allow the animals to open their shell valves and to resume pumping, algal cells were added to minimal concentrations of 15,000 cells ml⁻¹, but algal cells did not exceed 25,000 cells ml⁻¹, thereby avoiding pseudofaeces production and inhibition of clearance rate (Hawkins et al. 2001). In February 2000, scallops were fed *Phaeodactylum tricornutum*, whereas in June 2000 scallops were fed *Isochrysis galbana*. This was necessary, given that the optimal temperature for growth of *P. tricornutum* is between 15°C and 20°C, but higher for *I. galbana*. Average diameters of *P. tricornutum* and *I. galbana* are almost identical (5.2 to 5.5 µm). To test for any effects of different algal species, CR was compared in acclimated scallops feeding on either species of alga at 15°C.

After a 5-min period to allow thorough mixing of the algal cells, a 20-mL sample was taken from each beaker. Four subsequent 20-mL samples were taken at 30 min intervals over a period of 2 h. Cell concentrations in each sample were determined using a Coulter Multisizer II With a 100-µm aperture tube.

The clearance rate by individual animals was calculated as follows (Coughlan 1969):

$$\text{Clearance rate (l h}^{-1}\text{)} = V \times (\ln C_0 - \ln C_t) / t$$

Where C_0 and C_t were the cell concentrations at the beginning and end of each time increment, V is volume of water, t is the time between C_0 and C_t . The maximum clearance rate of each scallop was calculated based on the greatest decline in cell concentration over a 1-h period. This avoids the inclusion of periods when individuals may be totally or partially closed.

Oxygen Consumption Rate

Following the measures of CR, scallops were transferred to closed glass vessels containing 5 L of filtered (0.45 µm) seawater, each with a magnetic stirrer. Three scallops were put into each vessel, which was sealed and placed in a temperature-controlled oven. Dissolved oxygen (DO) was measured using an oxygen meter (YSI-556). Twenty minutes were allowed for the scallops to acclimate, after which their siphons were open. Oxygen uptake was measured over the next hour. Prior to each DO reading, the stirrer was operated for 20 sec. Oxygen concentration was not allowed to fall below 70% of saturation. Rates of oxygen consumption (OCR; µmol h⁻¹) were measured in *Chlamys farreri* acclimated to 3°C, 5°C, 8°C, and 15°C, and 1 day after transfer when fully acclimated from 15°C to 23°C, 25°C, and 27°C. At each temperature, declines in oxygen concentration were corrected for that consumed by the oxygen meter, using values measured in

a control vessel without scallops. At the end of the experiments the dry weight of each individual animal was determined after drying at 60°C for 48 h.

Weight Corrections

To standardize findings, rates were corrected for an equivalent individual of 1 g dry soft tissue using $Y_s = (W_s/W_e)Y_e$, where Y_s is physiologic rate of activity of a standard animal, W_s is the dry weight of standard animal (1g in this study), W_e is the dry weight of the experimental individual, Y_e is the uncorrected rate. B-values of 0.62 and 0.75 were used for CR and OCR, respectively, according to previous measures that confirm theoretical expectations in *C. farreri* (Lu et al. 2000, Hawkins et al. 2001).

Statistical Analyses

Weight-corrected CR and OCR were analyzed by ANOVA (one-way analysis of variance), with temperature as the independent variable. Pair-wise comparisons of the clearance rate at different temperatures were compared using Tukey's HSD multiple comparison test.

Model Predictions

Our dynamic model of responsive feeding, metabolism and growth in *C. farreri* cultured in Sanggou Bay (Hawkins et al. 2002) was adapted using findings presented here to further elucidate the effects of temperature on growth and possible mortality. As described by Hawkins et al. (2001), the correction factor used in this model for temperature effects on acclimated filtration rate (TEF; fraction) was derived from the Gaussian curve describing how CR varied with temperature that we document below (refer Results), as follows:

$$\begin{aligned} \text{TEF} = & (234.7 + (7.174 \times (6.283)^{0.5})) \times \text{EXP}(-0.5 \\ & \times ((-22.214) + 7.174)^2) + (234.7 + (7.174 \times (6.283)^{0.5})) \\ & \times \text{EXP}(-0.5 \times ((12 - 22.214) + 7.174)^2). \end{aligned}$$

Here, to predict responses in nonacclimated scallops, we instead implemented a modified TEF_{NA} based upon both the Gaussian and polynomial curves reported later (refer Results), describing how CR varied with temperature in both acclimated and non-acclimated scallops, respectively, as follows:

$$\begin{aligned} \text{TEF}_{\text{NA}} = & ((234.7 + (7.17 \times (6.283)^{0.5})) - \text{EXP}(-0.5 \\ & \times ((\text{Water_temperature} - 22.2) + 7.17)^2)) + ((234.7 \\ & + (7.17 \times (6.283)^{0.5})) \times \text{EXP}(-0.5 \times ((12 + 22.2) \\ & + 7.17)^2)) \times ((-18.059 + (3.078 \\ & \times \text{Water_temperature}) + (0.084 \\ & \times \text{Water_temperature} \times \text{Water_temperature})) \\ & + (-18.059 + (3.078 \times 15) + (-0.084 \times 15 \times 15))). \end{aligned}$$

To predict effects on metabolism in scallops subjected to abrupt increases in temperature, we continued to use the existing model correction factor reflecting a positive logarithmic dependence of

oxygen consumption upon temperature as documented later (refer Results), and which confirms a similar relation established previously at temperatures of up to 30°C (Lu et al. 2000, Hawkins et al. 2002).

The model with revised TEF_{NA} was run for 1 day, starting on Julian day 133 when the natural seawater temperature observed in Sanggou Bay was 15°C. Repeated simulations imposed stepwise increments in seawater temperature, each from 15°C, at 1°C intervals that ranged up to 30°C. Each of those runs used the same initial values of dry soft tissue weight (0.09 g), shell dry weight (0.64 g), shell energetic content (188 joules), and soft tissue energetic content (1717 joules), as had been predicted and validated for acclimated scallops, including the same data describing food availability and composition. By these means, we have predicted nonacclimated growth responses using our fully dynamic model in scallops subjected to abrupt stepwise increases in temperature from 15°C at 1°C intervals up to 30°C.

RESULTS

Comparison of CR When Feeding Upon Different Algal Species

Results from all experimental measures of CR ($l\ h^{-1}\ g^{-1}$), as outlined in Table 1, are illustrated in Figure 1. Average CRs in *C. farreri* feeding on different algal species *Isochrysis galbana* ($10.5\ L\ h^{-1}\ g^{-1}$) or *Phaeodactylum tricornutum* ($8.0\ L\ h^{-1}\ g^{-1}$) at 15°C were not statistically significant ($F = 3.31$, $df = 29$, $P > 0.05$). All measures derived for each algal species were therefore combined.

Effects of Temperature Change on Clearance Rate

In acclimated scallops, average CR increased with temperature from practically zero at 1°C to a measured maximum of $16.3\ l\ h^{-1}\ g^{-1}$ at 25°C, decreasing to around $7\ l\ h^{-1}\ g^{-1}$ at temperatures from 27°C to 30°C, but with greater individual variation. This increased variation in CR between individual scallops was because some animals had virtually ceased to feed at the higher temperatures.

The line of best fit describing CR in *C. farreri* that had been fully acclimated under standardized conditions of food availability varied with temperature (T ; °C) was a Gaussian curve illustrated in Figure 1 and described by the following equation:

$$CR = (234.7 \div (7.17 \times (6.283)^{0.5})) \times \text{EXP}(-0.5 \times ((T - 22.14) \div 7.174)^2),$$

where adjusted $r^2 = 0.85$, residual $df = 9$ and $P < 0.000001$ (from Hawkins et al. 2002).

Compared with acclimated scallops, there was little difference in average CR among nonacclimated animals that had been subjected to abrupt changes in temperature from 15°C to either 20°C ($F = 0.88$, $df = 29$, $P > 0.05$) or 23°C ($F = 0.23$, $df = 29$, $P > 0.05$) (see Fig. 1). However, average CR in nonacclimated scallops decreased significantly at temperatures both above and below this range, as represented by a polynomial curve illustrated in Figure 1 and described by the following equation:

$$CR = -18.055 + (3.077 \times T) - (0.084 \times T^2),$$

where adjusted $r^2 = 0.76$, residual $df = 23$ and $P < 0.01$.

The result was that average CRs at 25°C and above were significantly lower in nonacclimated than acclimated scallops (e.g., at 30°C, $F = 9.84$, $df = 23$, $P < 0.01$).

In scallops that had been acclimated at 30°C, and then transferred to 23°C, the average CRs determined after 1 and 4 days at 23°C (4.76 ± 1.90 and $6.51 \pm 4.79\ L\ h^{-1}\ g^{-1}$ respectively) were similar ($F = 0.12$, $df = 16$, $P > 0.05$), but significantly lower than among scallops acclimated at 23°C ($10.19 \pm 5.16\ L\ h^{-1}\ g^{-1}$) ($F = 7.65$, $df = 18$, $P < 0.05$; and $F = 8.29$, $df = 17$, $P < 0.05$; respectively).

Effects of Temperature Change on Oxygen Consumption

Average rates of oxygen consumption (OCR; $\mu\text{mol}\ h^{-1}\ g^{-1}$) in scallops acclimated at 3°C, 5°C, 8°C, and 15°C, and in nonacclimated animals transferred from 15°C to 23°C, 25°C, and

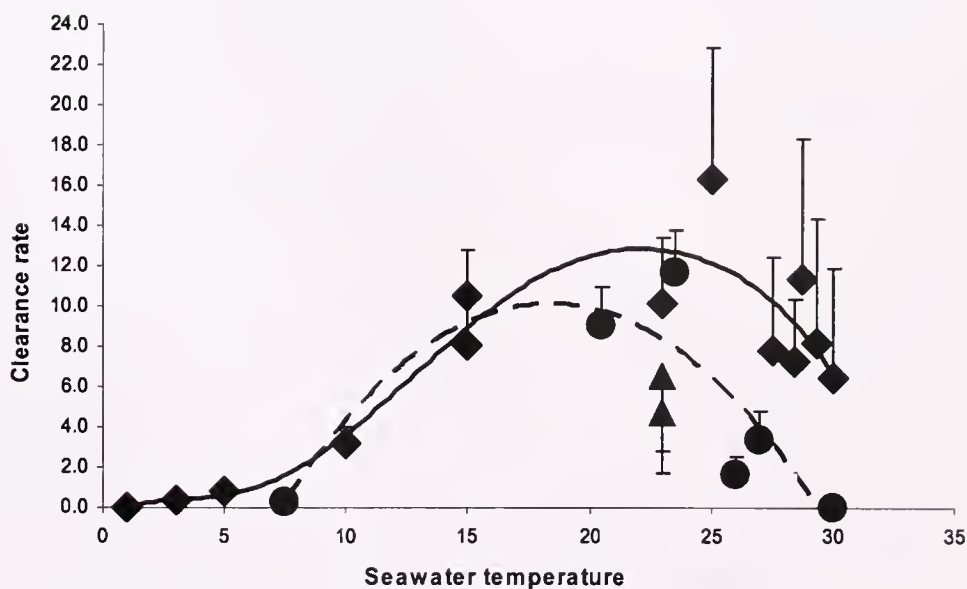


Figure 1. Clearance rate (CR, $l\ h^{-1}\ g^{-1}$) in *Chlamys farreri* acclimated to different seawater temperatures (solid line, diamonds), 1 d after transfer when fully acclimated from 15°C to other temperatures (dashed line, circles), and either 1 or 4 d after further transfer from 30°C to 23°C (triangles). Equations describing fitted curves are given within the Results. Values represent the average, and error bars ± 2 SE.

27°C, are illustrated in Figure 2. Whether in acclimated or in nonacclimated scallops, temperature had significant positive effects on OCR, which rose from $19.4 \mu\text{mol h}^{-1} \text{g}^{-1}$ at 3°C to $37.5 \mu\text{mol h}^{-1} \text{g}^{-1}$ at 15°C ($F = 38.65$, $df = 9$, $P < 0.01$), and then from $37.5 \mu\text{mol h}^{-1} \text{g}^{-1}$ at 15°C to a maximum of $88.1 \mu\text{mol h}^{-1} \text{g}^{-1}$ at 27°C ($F = 62.76$, $df = 9$, $P < 0.01$). Overall sensitivity of OCR to combined temperature increases as represented by Q_{10} value according to Bayne and Newell (1983) was $Q_{10} = (88.1/19.4)^{(10/(27-3))} = 1.9$. These findings are consistent with Q_{10} values of about 2, indicating approximate logarithmic increases in OCR with temperature reported in *C. farreri* and other shellfish species (e.g., Widdows 1973, Bayne & Newell 1983, Lu et al. 2000).

Effects of Temperature Change on Net Energy Balance

Figure 3 illustrates average seasonal changes in seawater temperature and suspended chlorophyll *a* ($\mu\text{g l}^{-1}$) measured at seven sampling sites throughout Sanggou Bay, China, and presents scallop growth both in terms of shell length (cm) and net energy balance (NEB; $\text{J h}^{-1} \text{g}^{-1}$), as predicted by our dynamic model simulating the traditional culture scenario. It shows how predicted shell length increased from 2 cm following seeding onto longlines in April to reach marketable sizes of more than 6 cm during the following October, according to historical practice and records (Hawkins et al. 2002). These predictions are for fully acclimated scallops, when NEB only becomes negative from December onwards, at a time when food availability measured as chlorophyll *a* abundance is low, and when temperatures fall below 8°C (see Fig. 3).

Figure 4 illustrates output from our revised model, predicting NEB in scallops 1 day after being subjected to a range of increased temperatures, where scallops had previously been acclimated at 15°C, and those increases in temperature were at 1°C intervals from 15°C up to 30°C. Our simulations indicate that maximal NEB would occur following exposure to 20°C, becoming negative following exposure to temperatures above 26°C. Energy losses of 60 J d^{-1} predicted following exposure to 30°C in scallops of this size (2 cm SL containing 1717 J within soft tissues) are equivalent to 3.5% of their soft tissue energy d^{-1} .

DISCUSSION

Bivalve feeding and metabolism is highly responsive to environmental conditions that include food availability (Newell & Shumway 1993, Iglesias et al. 1992, Hawkins et al. 1996, Hawkins

et al. 2001) and temperature (e.g., Bayne & Newell 1983, Navarro et al. 2000). An ability to acclimate to temperature change has been widely reported (Schulte 1975, Widdows et al. 1979, Bayne & Newell 1983, Navarro et al. 2000, Wang et al. 1999). In respect of feeding, this is confirmed for *C. farreri* by the differences observed here (see Fig. 1). Further, despite being uncertain that scallops were fully acclimated following each temperature change, our findings confirm a hyperbolic relation between acclimated CR and temperature, as reported in other species (e.g., Bougrier et al. 1995) (see Fig. 1). Significant reductions in CR were observed towards the extremes of temperature studied here, particularly when the change was made abruptly. This is of particular ecological significance for *C. farreri* in northern China, where the natural range of seawater temperature in areas used for the culture of scallops is much broader (1°C to 30°C) than most other regions of the world (Saxby 2002).

Our findings indicate that approximately 20°C to 25°C is the optimal temperature range for feeding, temperatures above this resulting in reduced rates of feeding and thus, by inference, the general health of scallops both in the short and long-term. This is borne out by our model predictions, suggesting that maximal NEB may occur at around 20°C, and that temperatures above 26°C, particularly if experienced abruptly, lead to reduced feeding, increased oxygen consumption and negative NEB (see Figs. 2, 3, 4). CR in animals acclimated above 26°C was not only reduced, but also variable between individuals (see Fig. 1). This may be interpreted as either a short-term survival strategy to reduce energy expenditure, or a differing susceptibility to the high temperatures causing some individuals to cease feeding completely. Further to this, CR in scallops acclimated at 30°C and then transferred to 23°C, did not recover to values in animals acclimated at 23°C, for at least 4 d. Further experiments are needed to confirm whether physiologic competence in respect of CR would have recovered over a longer period. If not, we might conclude that although exposure to temperatures of 27°C to 30°C was not lethal in the short-term, irreparable physiologic harm may have resulted in reduced fitness.

Our weight-corrected oxygen consumption rates of between approximately 20 and $100 \mu\text{mol g}^{-1} \text{h}^{-1}$ cannot easily be compared with previously published values in *C. farreri*; due to differences in methodology and units, previous values being expressed per individual, or in terms of wet weight (Wang et al. 1997, Wang et al. 1999, Yuan et al. 2000). However, rates reported here seem broadly comparable with those in previous studies, including those for other shellfish species (Haure et al. 1998, Bougrier et al. 1995, Aldridge et al. 1995, Taylor et al. 1995, Gunasingh et al. 2002, Gouletquer et al. 1999, Martinez et al. 1995).

Instead of calculating NEB using the simpler traditional measure of "scope for growth" according to Bayne and Newell (1983), we used our dynamic growth model for the scallop *Chlamys farreri*, thereby taking into account functional interrelations such as the influence of feeding rate upon absorption efficiency and a dependence of energy losses upon absorption rate (Hawkins et al. 2002). This model has been validated through both direct observations and records of growth as described by Hawkins et al. (2001). Simulations illustrated here indicate that in scallops acclimated to natural measured variations in temperature in Sanggou Bay, negative growth may occur from December onwards, when food availability is low, and temperatures are less than 8°C (see Fig. 3).

However, adaptation of that model in this study has shown that in nonacclimated scallops subjected to a major change in tempera-

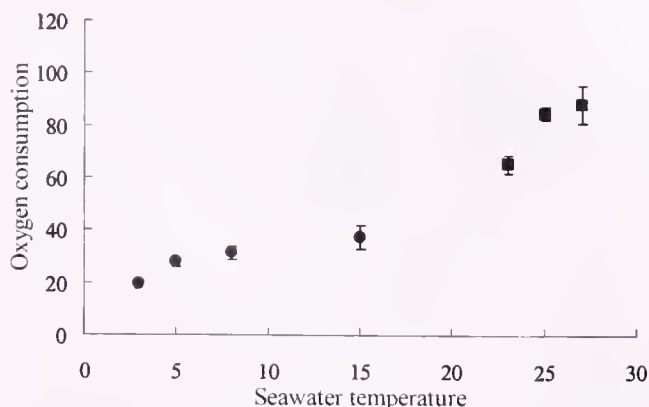


Figure 2. Oxygen consumption (OCR, $\mu\text{mol h}^{-1} \text{g}^{-1}$) in *Chlamys farreri* acclimated to different seawater temperatures (circles), and one day after transfer when fully acclimated from 15°C to other temperatures (squares). Values represent the average, and error bars ± 2 SE.

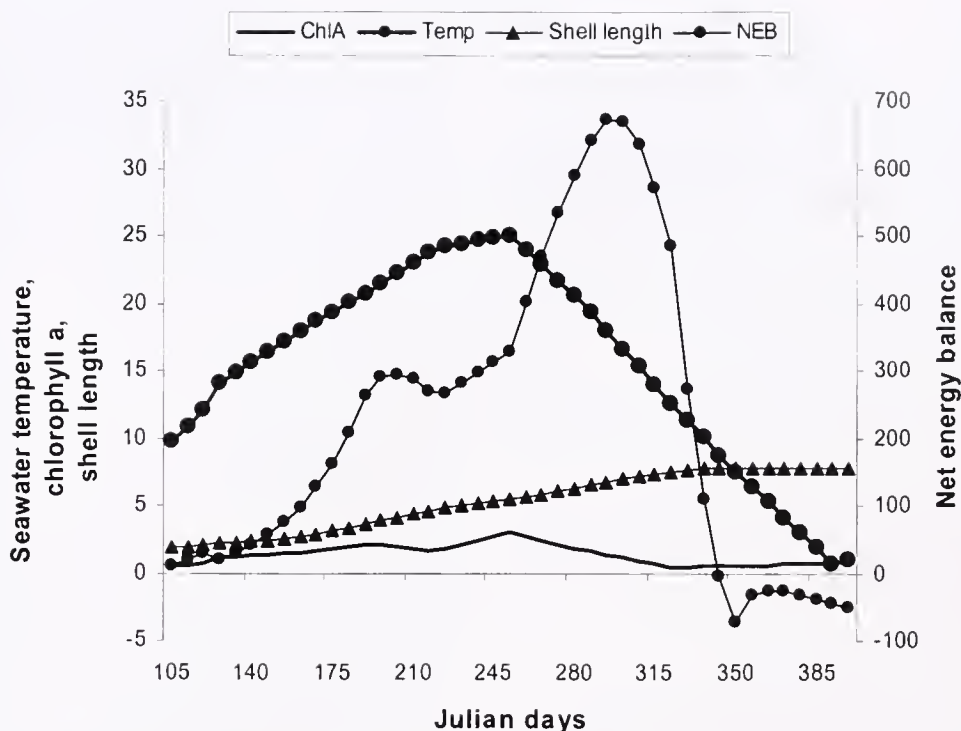


Figure 3. Growth in *Chlamys farreri* predicted both in terms of shell length (cm) and net energy balance (NEB, $\text{J h}^{-1} \text{g}^{-1}$) according to our dynamic model, in response to average seasonal changes in seawater temperature and suspended chlorophyll a ($\mu\text{g l}^{-1}$) measured at seven sampling sites throughout Sanggou Bay, China (Hawkins et al. 2002).

ture from 15°C to 25°C and above, CR may decrease dramatically, associated with increased OCR and negative NEB. These sublethal changes in temperature may occur naturally in Sanggou Bay during summer when temperatures above 25°C are common. For example, in recent summers, high temperatures of approximately 27°C lasted for several days along the coast of Shandong Province, especially in 1997. It has also been reported that the *El-nino* phenomenon elevated near-shore seawater temperatures abruptly by from 2°C to 5°C to as high as 29°C (Gao & Wang 1998, Li & Mou 1999, Shao et al. 1996, Shang et al. 1998). These high tempera-

tures or large changes in temperature may contribute significantly to severe physiologic stress. When compounded with other adverse environmental conditions, such as low food availability, pollution, toxic algal blooms or, a disease vector made more abundant or virulent by high temperature, increased mortality will inevitably result.

ACKNOWLEDGMENTS

The authors thank two anonymous referees for their helpful suggestions.

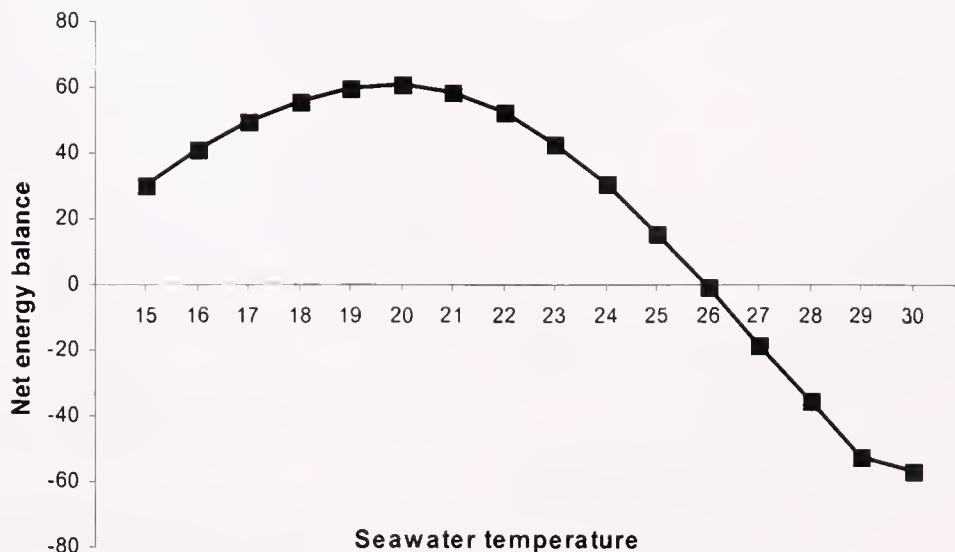


Figure 4. Growth predicted using our dynamic model adapted to simulate energy balance (NEB, $\text{J h}^{-1} \text{g}^{-1}$) in *Chlamys farreri* one day after being subjected to a range of increased seawater temperatures, where scallops had previously been acclimated at 15°C, those increases in temperature being at 1°C intervals from 15 up to 30°C.

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REPRODUCTION OF THE LION'S PAW SCALLOP *NODIPECTEN SUBNODOSUS* SOWERBY, 1835 (BIVALVIA: PECTINIDAE) FROM LAGUNA OJO DE LIEBRE, B.C.S., MÉXICO

MARCIAL ARELLANO-MARTÍNEZ,^{1,*} BERTHA PATRICIA CEBALLOS-VÁZQUEZ,¹
MARCIAL VILLALEJO-FUERTE¹, FEDERICO GARCÍA-DOMÍNGUEZ¹,
JUAN FELIX ELORDUY-GARAY,¹ AARÓN ESLIMAN-SALGADO² AND ILIE S. RACOTTA³

¹Centro Interdisciplinario de Ciencias Marinas, Instituto Politécnico Nacional Apartado Postal 592, La Paz, B.C.S. 23000, México; ²Reserva de la Biosfera el Vizcaíno, Guerrero Negro, B.C.S. México;

³Centro de Investigaciones Biológicas del Noroeste, Apdo. Postal 128, La Paz, Baja California Sur 23000, México

ABSTRACT Knowledge of the reproductive cycle of marine invertebrates of economic importance is essential for management of natural stocks and to initiate aquaculture activities. The reproductive biology of *Nodipecten subnodosus* from Laguna Ojo de Liebre, B.C.S., Mexico, was studied during a period of 25 mo. *N. subnodosus* is a functional hermaphrodite in which the follicles contain gametes of only one sex and remain separated from those containing the gametes of the other sex, with female follicles being dominant. Histologic evidence revealed that the lion's-paw scallop may remature at least once during the reproductive season. The temperature, analyzed as a single factor, could be important in the initiation of gametogenesis and/or as a spawning stimulus. The reproductive cycle shows a clear seasonality related to the water temperature, which is supported by the temporal variation of the oocyte diameter and by a significant positive correlation between GSI and temperature. The inactive period occurs from December to March, with low water temperature (16°C to 17.5°C). The gametogenesis started when the water temperature reached 18°C (April). The maximum maturation (90% to 100% of organisms) was reached in August when the temperature was highest (23.5°C). Spawning occurred in September when water temperature decreased. A slight reproductive activity during the cooler months (December to March) was observed. But, during 2002 the reproductive activity in that period was more pronounced and the slight spawning activity was extended to May. This behavior was in accordance with temperature anomalies that occurred in 2002. Then, *N. subnodosus* is potentially capable of spawning throughout the year if the environmental conditions are favorable. The length at first maturity obtained for *N. subnodosus* was 135-mmSL.

KEY WORDS: reproductive cycle, scallop, gonadosomatic index, histology, *Nodipecten*

INTRODUCTION

There are over 30 species of pectinids at the Mexican Pacific littoral (Keen 1971). However only three are considered as commercially important: the Pacific calico scallop *Argopecten ventricosus*, the Vogde's scallop *Euvola vogdesi*, and the Lion's paw scallop *Nodipecten subnodosus*. The lion's paw *N. subnodosus* inhabits shallow coastal channels with strong currents from Laguna Ojo de Liebre and the Gulf of California to Peru, (Keen 1971). It is one of the most important fisheries in the region, but indiscriminate harvesting has resulted in the depletion of most natural beds, and nowadays the exploitation is based solely on a natural population located in Laguna Ojo de Liebre B.C.S., México. Several institutions are now studying the natural history and developing technologic alternatives for this species (Morales-Hernández & Cáceres-Martínez 1996, Barrios-Ruiz et al. 2003, Racotta et al. 2003, Arellano-Martínez et al. 2004).

The knowledge of the reproductive cycle of marine invertebrates of economic importance is essential for management of wild populations and to initiate aquaculture activities. Documentation on the reproductive biology of *N. subnodosus* is scarce. Reinecke-Reyes (1996) reported the spawning season and reproductive cycle on the basis of a 10-month study in Laguna Ojo de Liebre, B.C.S. and using lion's paw scallops from the same locality, Gutiérrez-Villaseñor & Chi-Barragan (1997) made a laboratory experiment of reproductive conditioning. Recently, Racotta et al. (2003) studied the biochemical composition, growth, and length of first reproduction of cultured lion's paw scallops in Bahía Magdalena, B.C.S., and Arellano-Martínez et al. (2004), studied the relation-

ships between the nutrient storage cycle, reproductive activity, and available food of the same species in Laguna Ojo de Liebre, B.C.S.

We document here a detailed description of the gonad developmental stages of *N. subnodosus* and the reproductive cycle and its relation with temperature over 2 years in Laguna Ojo de Liebre, B.C.S., Mexico. Additionally, the length at first maturity was determined.

MATERIALS AND METHODS

From December 2000 to December 2002, 18 to 48 specimens per month of lion's paw scallop *N. subnodosus* were randomly collected by semiautonomous diving at 4–10 m depth from a wild population in the Laguna Ojo de Liebre, B.C.S., Mexico (27°35'N and 27°55'N and 113°50'W and 114°20'W). At the same time, bottom water temperature was recorded.

From each scallop the shell length was measured with a caliper at 1-mm resolution. The organisms were dissected and the total soft body and gonad wet weight to the nearest 0.1 g were registered. Gonad tissue was fixed in a buffered 10% formaldehyde solution for 24 h. A standard cross section of approximately 0.5 cm was cut in the middle part of the gonad, where both ovary and testis were included. Gonad samples were then dehydrated using a series of ethanol solution of increasing concentration, cleared in Hemo-De, and embedded in paraplast. Sections of 7-µm thickness were cut on a rotary microtome and placed on slides. Then, tissue was processed using a series of decreasing ethanol solution and stained with hematoxylin and contrasted with eosin (Humason 1979).

To analyze the reproductive process, gonad preparations were microscopically examined and assigned to a developmental stage following categories used previously for the same species (Reinecke-Reyes 1996): undifferentiated, developing, ripe, spawning,

*Corresponding author. E-mail: marellam@ipn.mx

and spent. The description of each stage of gonad development was complemented with our own observations. As a tool for the description of the reproductive cycle, the monthly relative frequencies of all gonad developmental stages were calculated.

The reproductive condition of specimens analyzed through histologic study was further examined by calculating a gonadosomatic index (GSI), as a ratio of the gonad wet weight to the total soft body wet weight (Sastry 1970). To determine the reproductive cycle and the GSI variation, only adult scallops (51–181 mm of shell length) were considered.

Additionally, the diameters of at least 100 oocytes per scallop were measured, using the SCAN PRO software (Version 5.0, Systat Software, Inc., Richmon, CA) and digitalized images of histologic sections. Only oocytes sectioned through the nucleus and with visible nucleolus were individually traced with the pointer and the major and minor axis lengths were automatically measured by the software. Then an average of both dimensions was calculated and it was considered the estimated mean diameter, which was calculated monthly. According to the criteria of Grant & Tyler (1983) individuals with few measurable oocytes and extensive phagocytosis ("spent" specimens) were not considered.

To assess the length at first maturity the cumulative relative frequencies of all mature scallops, excluding the undifferentiated, were calculated by shell length interval (5 mm). The length interval with 50% of mature scallops was considered as the length of first maturity (length at which lion's paw reach the maturity by first time) in the population (Somerton 1980).

One-way ANOVA followed by mean comparisons *post-hoc* Tukey test were made to assess significant differences in GSI and oocyte diameter between months. The differences in GSI between the same months of different years were evaluated by a *t*-test. A Spearman-rank correlation analysis was used to investigate the relationship among reproductive activity (GSI) and water temperature. Because GSI values are percentages, before applying the analysis they were arcsine transformed (Zar 1996) to reduce the dependence of the sample variance on the mean and to normalize the data distribution. STATISTICA for Windows (V. 6.0) was used for all analyses. The level of significance (α) was preset at 0.05.

RESULTS

Gonadic Structure

The lion's paw *N. subnodosus* is a functional hermaphrodite, in which both male and female gametes become ripe at the same time, although not necessarily they spawn at the same time. The gonad is not embedded in the visceral mass, and it is possible to differentiate the male from the female gonadic tissue from the outside. Macroscopically, the ovary portion has color ranges from a pale cream to dark orange, whereas the testis portion goes from creamy to beige, both varying with the degree of ripeness. The ovary portion, occupies the largest proportion of the gonad ($\approx 60\%$). The testis is located at the margin of the gonad from its origin (in the digestive gland) running anteriorly to the tip of the gonad.

Microscopically, the male and female tissues are organized in separated follicles and in different regions. However, the separation is incomplete, with small masses of spermatid tissue among the ovarian follicles or some ovarian follicles in the spermatid portion of the gonad.

Gonadal Developmental Stages

The histologic development of the gonad was simplified into 7 stages and their characteristics are as follows:

Undifferentiated

Scallops with little or no follicular material present and total absence of gametes. Connective tissue is abundant, containing different types of granulocytes and phagocytes. Externally the gonad is small and pale cream colored.

Early Development

This is the stage of gamete proliferation, characterized by the expansion of follicles, which are irregular in shape and contain oogonia (between 13 and 22 μm in diameter) or spermatogonia attached to the follicle wall. Interfollicular connective tissue decreases at this stage. A central lumen is present in each follicle. Externally, the gonad acquires a pale orange (ovary) and cream (testis) colors.

Late Development

A reduction of gonias and an increase of mature gametes occur in both sexes. As development proceeds, spherical vitellogenic oocytes (45 μm average diameter) and spermatozooids are more numerous. Spermatoocytes and spermatids are restricted to lining the follicular walls, which have become increasingly thinner. Some ripe gametes appear in the central lumen. Externally, the gonad acquires more consistency and the female orange color and male cream color are darker as the ripeness proceeds.

Ripe

Female follicles are distended and densely packed with mainly polygon-shaped oocytes. The oocytes reach their maximum size (51 μm average diameter). In male follicles dense masses of spermatozooids are located in the central lumen. In this stage, connective tissue is almost absent. Externally, the gonad is turgescence and the ovary portion acquires the characteristic dark orange color, whereas the testis portion is beige.

Partially Spawning

Variable quantities of follicles are partially or totally empty, depending on the degree of advance of the spawning. In some cases, very small previtellogenic oocytes or spermatoocytes are embedded in the follicle wall at the periphery of empty follicles. Externally, the color is similar to the ripe stage but the turgescence is lost. Gonads in a state of rematuration can be also assigned to this stage. These gonads are entering a new breeding cycle after their main spawning (Fig. 1a).

Spent

Gonad with ruptured follicles. Few residual oocytes and spermatozoa being phagocytized. There is no evidence of active oogenesis or spermatogenesis. The external appearance of the gonad is flaccid and pale orange or cream colored.

Reabsorption

This stage was observed in gonads with the micro and macroscopic characteristics of the late development stage, but with large quantities of vitellogenic oocytes or spermatozooids in reabsorption



Figure 1. Photomicrographs of a) ovary portion in rematuration, b) and c) reabsorption stage; b) ovary portion and c) testis portion. Numerous phagocytes (arrows) (macrophage hemocytes) between and within the follicles. Abbreviations: DO, developing oocytes; RO, Residual oocytes. Scale bar = 50 µm.

process (Fig. 1b,c). Numerous phagocytes were observed (macrophage hemocytes) between and within the follicles.

Reproductive Cycle

The reproductive cycle of *N. subnodosus* is shown in Figure 2. In December of all years, most scallops (between 82% to 100%)

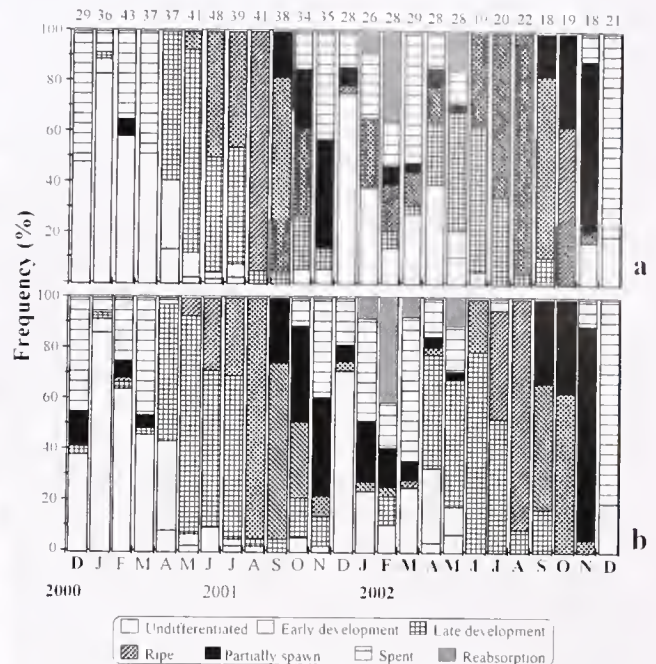


Figure 2. *Nodipecten subnodosus*. Monthly percent frequencies of the different gonad stages throughout the study period in Laguna Ojo de Liebre, B.C.S. México. a) females and b) males. Numbers at top indicate the sample sizes for each month.

were inactive (undifferentiated and spent stages). This reproductive quiescence was extended from January to March in 2001, but not in 2002 when gonads in re-absorption were observed for both sexes. In addition, some scallops presented female ripe gonads and partially spawned male gonads. Early development began in April as shown by the increase in numbers of discernible oögonia and small oocytes in the ovary region and the occurrence of spermatocytes or even spermatids in testis region. The late development stage proportion increased from April to July during both years, although the proportion of scallops in this stage was in general higher in April to May 2001 (60% to 90%) than for the same months during 2002 (25% to 55%). During the first year, ripe scallops were first observed from May (10%), reached a maximum proportion in August (near 100%), which progressively decreased thereafter to minimum values by December (5%). In the second year, in addition to the occurrence of reproductive activity in January and February previously described, the general pattern was delayed and shortened compared with 2001: initiation in June (22% to 40%), same peak in August (near 100%) and then almost no ripe scallops by November (5%). A massive spawning period was observed from September to November (25% to 75% of the population) in both years. In addition, a small proportion of partially spawned scallops were found in February to March 2001 (6.5%). As previously indicated a higher reproductive activity during these months was observed in 2002: ripe and partially spawned scallops were observed in January and their proportion in February to March (7% to 40%) was higher than in 2001.

Oocyte Diameter

The mean oocyte diameter (Fig. 3) showed significant differences among months (one-way ANOVA, $P < 0.01$) with a clear seasonality (*post hoc* Tukey test) during both years, with the large-

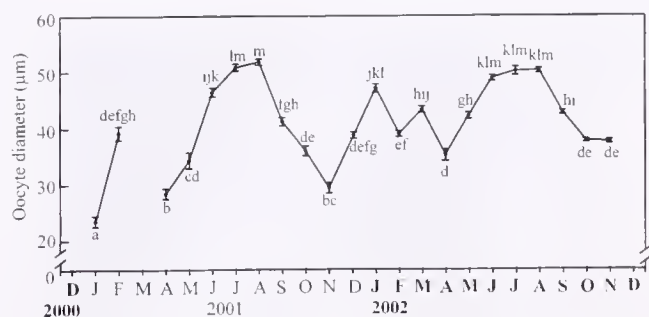


Figure 3. *Nodipecten subnodosus*. Temporal variation in oocyte mean diameter. Data were analyzed by 1-way ANOVA followed by Tukey test. Means not sharing the same superscript are significantly different. Bars correspond to standard errors.

est oocytes from June to August. In 2002 the seasonality was not so clear: in addition of the largest oocytes found from June to August, large oocytes were also observed in January and March. The minimum mean oocyte diameter (23.5 μm) was observed in January 2001, and the maximum (51 μm) was observed in August 2001. In December 2000, March 2001, and in December 2002 the scallops were spent or undifferentiated and thus oocyte diameter was not estimated, but very low values would be obtained in these months.

Gonadosomatic Index

GSI showed a marked seasonal pattern with significant differences (one-way ANOVA, $P < 0.01$) throughout the study period (Fig. 4). The GSI values were low from January to March of the first year (5.7% to 7.7%); and from January to May of the second year (5.9% to 8.3%). Values of the GSI increased gradually from April of the first year and from June of the second year. GSI reached the maximum value in August of both years (27.0% and 27.7% respectively), being significantly different from the other months. From September of both years, the GSI values decreased until the lowest values were reached in December (7.0%). Comparing the GSI values of each month between years, significantly lower values were obtained in 2001 than in 2002 for February, March, June, and July, whereas significantly higher values were obtained in 2001 than in 2002 for May and September ($P < 0.05$, t -test, Zar 1996).

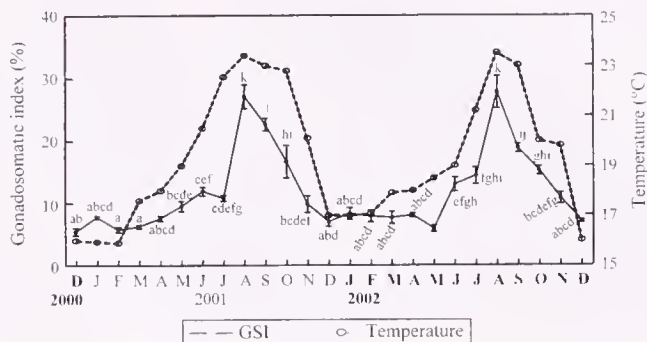


Figure 4. Temporal variation in water temperature in the Laguna Ojo de Liebre, B.C.S., and the temporal variation in the mean values of gonadosomatic index. GSI data were analyzed by 1-way ANOVA followed by Tukey test. Means not sharing the same superscript are significantly different. Bars correspond to standard errors.

Seawater Temperature

Seawater temperature varied seasonally, with its minimum values in January to February (15.9°C) (see Fig. 4). Temperature increased gradually from March 2001 and from February 2002, reaching its maximum values in August (23.5°C) of both years. Temperature values started to decrease in September in both years. Interannual variations of water temperature were registered (Table 1). The period of January to March 2002 was warmer and the periods of May to July and October to December 2002 were cooler than the same periods in 2001. A significant correlation between the GSI and seawater temperature ($R = 0.89$, $P = 0.000$) was found.

Length at First Maturity

The range in scallops shell length was 51-mm to 174-mm (130.3-mm average, 18.8-mm standard deviation). A logistic model was fitted to data on shell length versus maturity. Although some specimens may start their gonad development as small as 51-mm SL, the length at first maturity in *N. subnodosus* was found at 135-mm SL (Fig. 5).

DISCUSSION

The sexuality of pectinids ranges from strictly gonochorists to functional hermaphrodites (Coe 1945, Reddiah 1962), and there are 2 types of follicular structure in hermaphroditic specimens (Coe 1945, Reddiah 1962, Gaspar-Soria et al. 2002). In the type 1 the follicles contain gametes of only one sex, and remain separated from those containing the gametes of the other sex, whereas in the type 2 the male and female gametes are present in the same follicle. According to the earlier mentioned facts, the histologic analysis confirmed that *N. subnodosus* is a type 1 functional hermaphrodite, with female follicles being dominant. The most common form of hermaphroditism in pelecypods is the type 1 (Sastri 1979), although the occurrence of type 2 is frequent (Rose et al. 1990, Villalejo-Fuerte et al. 1996, Ceballos-Vázquez et al. 2000, Gaspar-Soria et al. 2002). However, the hermaphroditism is very complex in pectinids. For example, *Placopecten magellanicus* is considered to be a gonochoric species (Parsons et al. 1992), although some cases of type 2 hermaphroditism with dominance of male gametes have been reported from this species (Giguere et al. 1994). This indicates that pectinids may have individual variations

TABLE 1.
Interannual variation of water temperature.

Month	2000	2001	2002	Difference
January		15.9	17	1.1
February		15.9	17.3	1.4
March		17.6	17.9	0.3
April		18	18	
May		19	18.5	-0.5
June		20.5	19	-1.5
July		22.5	21.2	-1.3
August		23.4	23.4	
September		23	23	
October		22.8	20	-2.8
November		20.1	19.8	-0.3
December	16	17	16	-1.0

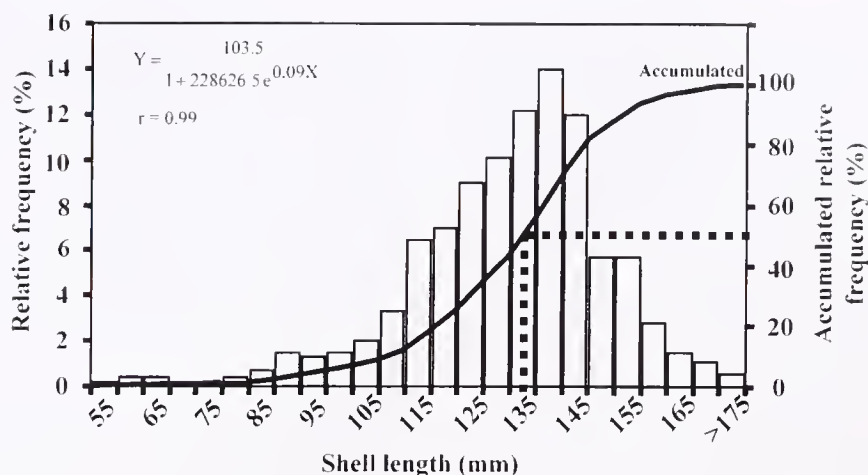


Figure 5. Length at first maturity of *Nodipecten subnodosus* from Laguna Ojo de Liebre, B.C.S., México.

in the expression of sexuality and probably different reproductive strategies.

Although the gonad of hermaphrodite scallops can produce both types of sexual cells at the same time, they are not usually discharged simultaneously, to avoid self-fertilization. Morales-Hernández and Cáceres-Martínez (1996) mentioned that the hermaphrodite scallops, first release the sperm and subsequently release the oocytes. In this study, we found gonads with the testis partially spawn or spent, together with a ripe ovary also suggesting that the sperm was released before the eggs. This behavior was observed during spawning induction for *N. subnodosus* under laboratory conditions although it depends on the method of induction (Ibarra personal communication, CIBNOR, La Paz, Mexico). Additionally, the histologic evidence revealed that *N. subnodosus* may remature at least once and possibly more, during the reproductive season because we frequently observed individuals in which one sex was in an advance developmental stage whereas the other sex was in an early development stage. Therefore scallops avoid the spent stage and they go directly to a new development after the first spawning to start on another breeding cycle. It is important to clarify that all of these scallops were found only after the main spawning period (when the rematuration of testis and ovary is asynchronous as a consequence of the alternate spawning by sex), and never at the start or end of the reproductive activity period. The presence of rematuration has been also reported for *Pinctada mazatlanica* (Sevilla 1969, Saucedo & Monteforte 1994) and *Argopecten ventricosus* (Villalejo-Fuerte & Ochoa-Báez 1993).

Gametogenic cycles are generally ruled by external environmental factors (i.e., temperature, photoperiod, food availability) that may trigger and synchronize the "timing" of the different stages (Lubet 1983, Gallardo 1989). It is well established that temperature is one of the most important environmental factors influencing the regulation of gametogenic cycle of mollusks (Giese 1959, Mann 1979, Sastry 1975, Giese & Kanatani 1987, Wada et al. 1995). In some scallops likely *Chlamys islandica* sudden increases in water temperature seem to be the final cue for stimulating maturation and spawning (Skreslet & Brun 1969, Giguere et al. 1994), or in other scallops such as *N. nodosus* a specific temperature (17.5°C) seems to increase sexual maturity and synchronize the reproductive behavior (Rupp et al. 1997). However, the effects of temperature on gametogenesis and spawn-

ing cycles of bivalves are complex, and depend on the overall reproductive strategy and the environmental temperature range (Marsden 1999). In this study, the reproductive cycle of *N. subnodosus* shows a clear seasonality related with the water temperature, which is supported by the temporal variation of the oocyte diameter and by the significant positive correlation between GSI and temperature. In a general context, the inactive period (undifferentiated and spent stages) occurs from December to March, with low values of water temperature (16°C to 17.5°C) and GSI (5.4–8.2). Gametogenesis started in April when the water temperature reached 18°C and continued during the warmer months. The maximum maturation (90% to 100% of organisms) was reached in August when the temperature was (23.5°C) and GSI (27.0 in 2001 and 27.7 in 2002) were highest.

On the other hand, the spawning of *N. subnodosus* occurred in September when water temperature and GSI decreases, and continued through November. This is in agreement with the highest spat concentration found within this period by García-Domínguez et al. (1992). The general reproductive pattern also agrees with previous studies in this species at the same locality (Reinecke-Reyes 1996, Arellano-Martínez et al. 2004) and at a southern locality (Racotta et al. 2003).

In addition to the general reproductive events analyzed earlier, the qualitative histologic observations revealed a slight reproductive activity during the cooler months (January to March of 2001), as indicated by the presence of reproductively active scallops in a low proportion (2.7% to 6.8%). Notwithstanding, during 2002 the reproductive activity in that period was more pronounced, with higher proportion of reproductively active scallops (3.4% to 26.9%) and in addition, the spawning was extended until May. This behavior was in concordance with the temperature anomalies occurred in 2002, which were higher than in 2001, and thus reinforces the influence of temperature on the gametogenic cycle of *N. subnodosus*. In this sense, Jaramillo and Navarro (1995) suggested that spawning could be induced by a combination of internal and environmental factors and that their interaction may vary seasonally, producing annual variations in onset and intensity of spawning. On the other hand, Brousseau (1987) mentioned that it is possible that some species could begin a facultative spawning if environmental conditions are favorable. An earlier, minor spawning is common in other species such as *Mya arenaria* (Brousseau 1987), *Pecten maximus*, *P. magellanicus*, in species of the genus

Chlamys (Thompson 1977), and in the particular case of *N. subnodosus* (Arellano-Martínez et al. 2004). However, it is important to stand out that only in this period of possible facultative spawning (January to May 2002), gonadal regression (reabsorption stage) was found. Similarly, a thermal stress has been shown to prevent or inhibit the egg release in *P. maxima* (Rose et al. 1990). In this sense, the massive lysis of gametes can be caused, among other factors, by unfavorable environmental conditions, thus stopping a sexual cycle, which in favorable conditions would end with spawning (Motavkine & Varaksine 1983, Dorange & LePennec 1989). Therefore, we believe that *N. subnodosus* is potentially capable of spawning throughout the year if the environmental conditions are favorable.

The bivalve production of gametes is also influenced (set in a seasonal context) by food availability (Bayne 1976, MacDonald & Thompson 1985, Jaramillo et al. 1993). It has been suggested that spawning of some scallops may be triggered by changes in temperature and/or phytoplankton blooms (DiSalvo et al. 1984, Villalaz 1994, Jaramillo et al. 1993, Arsenault & Himmelman 1998, Luna-González et al. 2000, Arellano-Martínez et al. 2004). Although in this study the food availability was not determined, the higher concentration of chlorophyll *a* (1.2 mg/L) in the Laguna Ojo de Liebre was found in September to October (Arellano-Martínez et al. 2004), just along with the spawning. It has been previously suggested that the spawning of *N. subnodosus* in Laguna Ojo de Liebre seemed to be triggered more by the phytoplankton abundance than by temperature (Arellano-Martínez et al. 2004), probably as a strategy to ensure food supply to the larvae (Newell et al. 1982). In this sense, Woll (1988) suggests that high temperature, although favoring maturation and spawning, might be less critical for a successful spawning than food availability. From the results of this study, it is now difficult to separate between the relative influences of temperature and food availability, but probably both factors play an important role.

Finally, no data are available so far concerning the length at first maturity of *N. subnodosus*. Our results indicate that the individuals of this species attain their first maturity at a shell length of 135-mm. However, histologic observation showed some individuals of *N. subnodosus* sexually mature at 51-mm SL. García-Domínguez et al. (1992) reported that this species grows 0.21 mm of shell height by day at the same locality, but in culture. This shows that this species becomes sexually mature at approximately 8 mo of age in Laguna Ojo de Liebre. Similarly, Racotta et al. (2003) reported, in culture conditions, maturing *N. subnodosus* scallops of 8 mo of age (55 mm SL, approximately) in Bahía Magdalena, B.C.S., México, a locality about 400 miles to the south.

In conclusion, lion's paw *N. subnodosus* from Laguna Ojo de Liebre, Mexico, is sensitive to water temperature changes and it behaves as many temperate species, maturing during the warmer months and spawning when temperature begins to decrease, with minimum or null reproductive activity during winter (December to March). This pattern agrees with the northern pectinid *Aequipecten tehuelchus* (Narvarte 2001). Villalaz (1994) mentioned that the temperature probably has a greater influence on reproduction in temperate than in tropical bivalves, because in the former, thermal fluctuations are higher.

ACKNOWLEDGMENTS

The authors thank Compañía Exportadora de Sal, Sociedad Cooperativa Pescadores Unidos de Guerrero Negro, Reserva de la Biosfera El Vizcaíno, and Instituto Politécnico Nacional for the grants (EDI and COFAA) and the authors. Marcial Arellano is a PhD student-fellow of CONACyT. Study supported by the following projects: CGPI- 20031573, 20038005, and SEMARNAT 2002-C01-0277.

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REPRODUCTION AND PATHOLOGY OF BLUE MUSSELS, *MYTILUS EDULIS* (L.) IN AN EXPERIMENTAL LONGLINE IN LONG ISLAND SOUND, CONNECTICUT

INKE SUNILA,^{1,*} LAWRENCE WILLIAMS,² STEVE RUSSO³ AND TESSA GETCHIS⁴

¹State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97,

Milford, Connecticut 06460; ²Jessie D., Inc., 68 Anchorage Drive, Milford, Connecticut 06460;

³Cornell University, 126C Valentine Place, Ithaca, New York 14850; ⁴Connecticut Sea Grant,

University of Connecticut, 1084 Shennecossett Road, Groton, Connecticut 06340

ABSTRACT An experimental longline was deployed in Long Island Sound, Connecticut to study the biologic feasibility of commercial culture of blue mussels, *Mytilus edulis*, in the area. Mussels were sampled monthly for 1 year starting 6 months after the seed settled. Samples were processed for histology and studied for gonad development to predict spawning peaks for future seed line deployments. There were 2 spawning peaks: a major one in May and a minor one in August. Gametogenic activity was detected throughout the year with developing gonads present in February and spawning specimens present from April to January. Samples were also examined for pathology to determine if there were diseases or parasites that could affect the operation. Mussels acquired heavy infections of the trematode parasite *Proctoeces maculatus* (over 60%) in September that lasted to the end of the year. Large areas of the mantle and visceral tissues were replaced with larval and adult parasites. Abscesses were formed with adult trematodes surrounded by massive aggregations of host hemocytes. Mussels reached sexual maturity and commercial size (60 mm) during their first 6 months after seed set. Two opposing forces drove the feasibility of the operation in this atypically southern location for mussel culture: exceptionally rapid growth, but also heavy infections by a trematode parasite enzootic to tropical and temperate waters. We conclude that the biologic potential for a commercial operation exists for a seasonal product that would use the window of opportunity for parasite-free full-grown mussels from the first winter after settlement until the next midsummer.

KEY WORDS: reproduction, pathology, longline, blue mussel, *Mytilus edulis*, *Proctoeces maculatus*, Long Island Sound

INTRODUCTION

Culture of blue mussels, *Mytilus edulis* (Linnaeus 1758) is a fast growing sector of shellfish aquaculture. Global aquaculture production of mussels has increased 20-fold (from less than 100 kilotons to over 1.3 million tons) during the past 50 y (McLeod 2002). Mussel production has been dominated by Europe (mainly Spain, Italy, the Netherlands, and France) and China with combined output exceeding 90% of world production (McLeod 2002). Mussel culture, still a diminutive player on a global scale, in North America has been growing constantly. The market leader is Prince Edward Island (PEI), Canada with an estimated 50% of the North American mussel market. Submersible longline technology is used in PEI (McDonald et al. 2002).

Three mussel species are cultured in the USA: *Mytilus edulis* in New England, *M. trossulus* (Gould 1850) in Alaska, both *M. galloprovincialis* (Lamarck 1819) and *M. trossulus* in Washington State, and *M. galloprovincialis* in California. In the Northeast USA about half of the current production is still largely based on wild fishery (bottom harvest) and the other half coming from cultured product with a total of 7.3 million kg annually. Raft culture is used widely in Maine, the Northeast USA market leader in mussel production (King & Cortes-Monroy 2002, The Island Institute 1999). A pilot-scale mussel longline culture was established in 1999 at a site eight kilometers from the shore in the Gulf of Maine, New Hampshire, to study the feasibility of deep-water culture (Langan 2000, Langan & Horton 2002). A mussel longline was also established in Narragansett Bay, Rhode Island (Corayer 2003). Growing interest toward blue mussel culture is due to the easy collection of natural seed, relatively low operation costs and short production cycles. Unlike most bivalves mussels have a short shelf life, a problem that could be overcome with local production.

Mussel culture is usually based on collection of natural seed. Hatchery production of mussel seed is used only in Washington State, Australia, and China (King & Cortes-Monroy 2002). Because of the importance of accurate timing for deployment of seed collecting lines to catch mussel spat and avoid setting of fouling organisms, the reproductive cycle of *Mytilus* has been studied extensively. Studies from different European estuaries reported that spawning times differed between areas from March until July as well as annually. In general, spawning occurred later, was shorter, and had only one peak in the more northern latitudes compared with those areas further south. A second, less pronounced, spawning peak usually manifested itself later during the summer or fall in more southern areas (e.g., Bayne 1964, Chipperfield 1953, Kautsky 1982, Seed 1975, Sunila 1981, Thorarinsdóttir 1996).

A similar pattern was observed on the East Coast of North America (Thompson 1984). Mussels in Newfoundland spawned in late July. The reproductive cycle resembled that of Baltic mussels rather than mussels in central European locations. The reproductive cycle of *M. edulis* in Long Island Sound (LIS) has been studied as follows: Hrs-Brenko (1971) sampled in Milford, Connecticut (CT), LIS from March until July; Brousseau (1983) in Fairfield, CT, LIS for a period of 2 years; and Fell & Balsamo (1985) in the Thames River and at Branford, CT, LIS from May until June. These reports studying wild mussel populations concluded that spawning occurred in May to June. Newell et al. (1982) sampled several sites between Maine and Delaware Bay and reported that at one of the sites, Stony Brook, NY, LIS, spawning occurred from April to May.

When predicting accurate timing for setting seed collecting lines, the length of the pelagic phase of mussel larvae must be taken into consideration. Peak setting of mussel larvae in Milford Harbor generally occurred between June 29 to July 6 (Engle & Loosanoff 1944) but in late fall in the Thames River (Fell & Balsamo 1985). According to their observations, spawning and

*Corresponding author. E-mail: isunila@snet.net

setting occurred about 4 months earlier in the central shores of the Sound. Larval period is estimated as being 3–5 wk after which the peak settlement occurs (Seed 1969). However, unfavorable conditions could prolong it for up to 6 months (Nelson 1928, Lane et al. 1985). Postlarvae are capable of secreting a long drifting thread to remain pelagic, which enables the mussels to extend their distribution.

Massive mortalities of cultured *M. edulis* are generally not associated with disease outbreaks. Virus-like disease has been reported to cause mortalities (50% to 100%) of cultured green-lip mussels *Perna canaliculus* in New Zealand (Jones et al. 1996), but virus-associated granulocytomas in *M. edulis* in Denmark were not associated with mortalities (Rasmussen 1986). Marteiliasis (*Marteilia refringens*, usually a pathogen of *Ostrea edulis*) has been associated with mortalities of *M. galloprovincialis* in Spain (Villalba et al. 1993), but not in *M. edulis* in Brittany, France (Robledo et al. 1994a). Disseminated sarcoma has caused significant mortalities of cultured *M. trossulus* in Puget Sound, Washington but doesn't reach epizootic prevalences in *M. edulis* at different sites in the Atlantic (Elston et al. 1992). Figueras et al. (1991) sampled wild mussels from the coasts of New Jersey and Maine to provide preliminary baseline pathology data. They reported the presence of microsporidia *Steinhausia mytilovum*, an intracellular ciliate inside digestive cells, and trematodes *Proctoeces maculatus*. They also observed Rickettsiae-like prokaryotic inclusions in the digestive cells, haplosporidia-like protozoan and microcell-like organisms that resembled *Bonania*. The presence of these parasites was not reported to be associated with mortalities.

Even though *Mytilus* is a dominant species of the northern hemisphere, a key aquaculture species and part of the natural fauna in LIS (Weiss 1995), no efforts have been made to commercially grow the mussel in LIS. Several papers were published concerning the reproduction of *Mytilus* in LIS, but did not provide information about the actual spawning times. Fell and Balsamo's (1985) observations from Branford were based on sampling during 2 months, whereas Hrs-Brenko's (1971) studies covered 4 months. Newell et al. (1982) and Brousseau (1983) used stereology to assess reproductive status and expressed it as the "gamete volume fraction" which is the proportion of the mantle tissue that is composed of follicles containing developing or ripe gametes. This method doesn't include visual observation of gamete release, and pre and post spawning status can show similar values. There is a paucity of information concerning the pathologic status of mussels in LIS. Thus, an experimental mussel longline was deployed in LIS in the summer of 2001 to determine if there are diseases or parasites that could affect mussel culture in the area and to define spawning peaks for future seedline deployments.

MATERIALS AND METHODS

A longline for mussel seed collection was deployed in summer of 2001 on a shellfish lease in Milford, Connecticut. The longline is simply a long piece of rope that is fixed horizontally in the water column (Fig. 1). A horizontal head rope (30 m, 9.5 mm sinking rope) was submerged to 1.8 m below the surface at mean low tide and anchored at each end with concrete blocks (68 kg, 0.2 m × 0.3 m × 0.2 m). Water depth at the lease was 10 m. Vertical seed collecting lines were hung below the head rope at 1 m intervals and their weight was supported by a series of buoys. Three-meter long collecting lines were composed of alternating 6.4 mm Polyplus rope and 12.7 mm sinking rope. Two marker buoys were tied to the

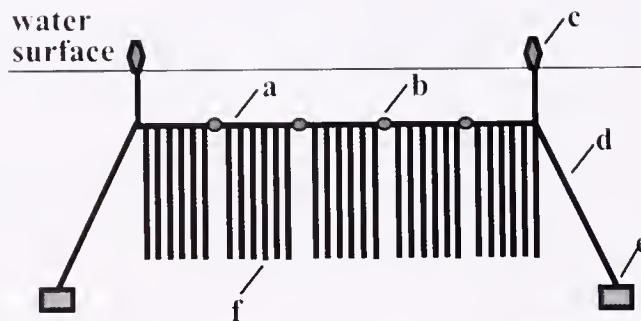


Figure 1. Design of longline system a = head rope, b = submersible buoys, c = marker buoys, d = anchor line, e = anchor, and f = seed collecting lines.

headline at the quarter- and three-quarter distance marks. Mussels were left on the seed lines for the grow-out period.

A random sample of 30 mussels was taken monthly from February 2002 to January 2003 to study the developmental stage of the gonads and pathology (a total of 360 mussels). Temperature was recorded at a distance of 300 m from the longline site in Milford Harbor (Table 1). In the laboratory, the mussels were measured with calipers (maximum length) and examined gross-macroscopically. Fouling organisms and the presence of pea crabs (*Pinnotheres maculatus*) were recorded and the pea crabs were removed at this point. Samples were fixed in Davidson's fixative and processed for pathology using standard histologic techniques. A 5- μ m thick paraffin section was stained with hematoxylin-eosin. The developmental stage was expressed by a gonad index (Chipperfield 1953). The developmental stage of the gonads was classified according to Seed (1969). Developing specimens were described by numerical scores 1 to 5 and spawning by values 4 to 1. A resting or immature gonad was defined with value 0. Gonad index varied from zero, when no sexual activity was detected, to five, when all the individuals were ripe. The number of mussels at each stage was multiplied by the numerical score of the stage and the sum was divided by the number of individuals in the sample to obtain a weighted gonad index.

TABLE 1.

Sampling times, average size of mussels ($n = 30$) collected, seawater temperature, fouling with *Crepidula fornicata* and the prevalence of *Pinnotheres maculatus* (% of specimens affected) in a mussel (*Mytilus edulis*) longline culture over the course of 1 year in Long Island Sound, Connecticut.

Sampling Time	Size (mm)	Temperature C	<i>Crepidula fornicata</i> %	<i>Pinnotheres maculatus</i> %
2.20.02	56	4.9	0	3.3
3.21.02	52	6.0	0	3.3
4.23.02	61	11.4	0	0
5.21.02	60	12.2	0	0
6.27.02	68	22.3	0	0
7.30.02	66	23.7	86.6	0
8.30.02	72	23.2	70	0
9.30.02	62	20.8	93	3.3
10.31.02	70	12.4	100	0
11.19.02	70	10.3	100	20
12.16.02	69	5.9	100	23.3
01.16.03	66	2.5	100	60.0

Classification of the Gonad Developmental Stages

Developmental Stage

0. Immature or already spawned, resting gonads. No germinal follicles are observed in the mantle and the sexes cannot be distinguished.
1. Ducts lined with germinal epithelium start to appear in the middle of the connective tissue in the mantles. Ripe gametes cannot be observed, but early stages of gametogenesis are present.
2. Gonoducts expand displacing the connective tissue. Ripe sperm and ova appear in the middle of the follicles, but the early stages such as spermatogonia, spermatocytes, and oocytes fastened to the germinal epithelium dominate.
3. The follicles are about half-filled with mature gametes, the remaining area consisting of early stages of gametogenesis. The ducts and follicles have expanded to fill about half of the mantle matrix.
4. Follicles have invaded almost the entire mantle. Cells undergoing gametogenesis can still be found in the margins of the ducts, but ripe gametes dominate.
5. Follicles are full of ripe gametes. Some gametocytes are still in the margins of the ducts (gamètes de réserves, Lubet 1957). Tightly compacted ova have an angular configuration.

Spawning Stage

4. The release of gametes from the mussel has begun. Follicles are still full of gametes, but their numbers in the lumina of the follicles have decreased and the ova have a spherical shape.
3. Follicles are half-filled with gametes. This can be distinguished from the previous developmental stage by the scarcity of developing cells.
2. Gametes fill less than half of the follicles. The ducts continue to shrink, whereas the connective tissue is expanding.
1. The follicles have disappeared almost completely. Some sperm and ova remain in the follicles. There may be hemocytes in the follicles phagocytizing residual gametes.

All pathologic changes were recorded for characterizing the health of the mussels. Additional pea crabs were observed in histologic sections and their number was added to the count obtained from gross-macroscopic examination to determine the actual prevalence.

RESULTS

Mussel seed set on the longline in July of 2001. Sampling for histologic examination started in February 2002 and ended in January 2003. The mussels were followed from 6 mo of age for a full year during the experiment. The size of the mussels did not increase significantly during the experiment (Table 1) indicating that the mussels had reached approximately their maximum size during their first 6 months after setting. During the first 5 months of sampling no fouling organisms were present, but after that mussels were covered with slipper shells, *Crepidula fornicata* (Table 1). Barnacles and bryozoa also attached to shells, but slipper shells covered these organisms to such an extent that the prevalences of the latter were not recorded. Concurrently with the invasion of slipper shells the lines were covered with hydroids (*Tubularia* ssp.) that were removed prior to sampling of the mussels. The mean prevalence of pea crabs (*Pinnotheres maculatus*) was

9.4% during the year. Their presence had a seasonal pattern with absence during the spring and summer months and a peak from November through January (see Table 1). Nine out of the total of 34 pea crabs (26%) were observed histologically only and were not noted during gross-macroscopic examination due to their small size. Most of the infestations were single pea crab/mussel associations, but one mussel was harboring 2 pea crabs, 1 female and 1 male.

Reproduction

Sex distribution was 43.3% females, 39.7% males, and 15.0% sterile or indeterminate. Seven (1.9%) were hermaphrodites with 5 having ova and sperm in separate follicles. In one of the specimens, half of the body was producing female gametes and the other half male gametes. Two specimens had compound follicles with both ova and sperm present.

In February and March genital tissue started to develop in the mantle (Fig. 2A, B), the outer surface of the digestive gland and the pericardium. Female gametes were fastened with a peduncle to the germinal epithelium during development. In ripe female fol-

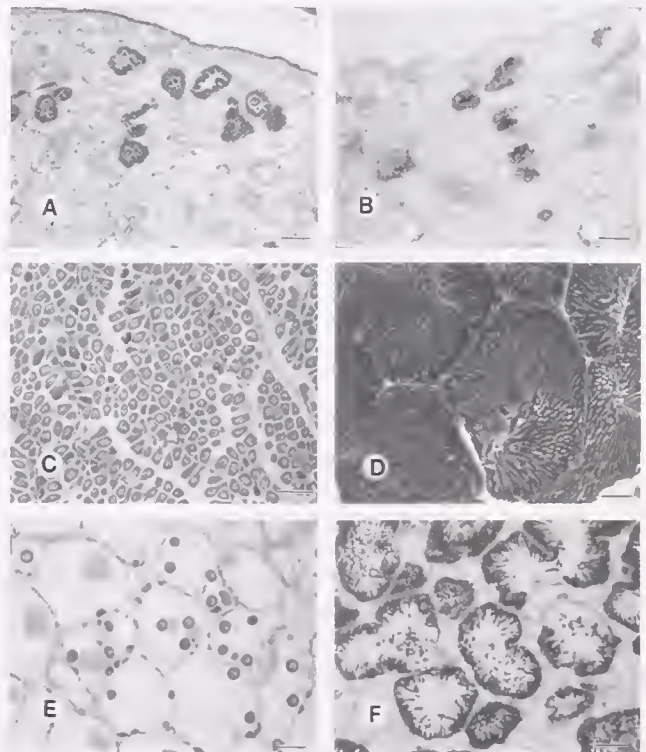


Figure 2. Reproduction of mussels (*Mytilus edulis*) in a long line culture in Long Island Sound, Connecticut. Hematoxylin-eosin stained sections. (A) Female; Developmental stage 1. Follicles with developing oocytes have appeared in the mantle tissue. (B) Male; Developmental stage 2. Follicles with germinal epithelium and some ripe sperm can be seen in the mantle tissue. (C) Female; Developmental stage 5. Mantle is completely invaded with follicles. Tightly compacted ova have angular configurations. (D) Male; Developmental stage 5. Mantle is completely invaded by follicles. There are still some gametocytes present but mature sperm fills the follicles. (E) Female; Spawning stage 2. Ova are being released from the gonad in the seawater. Follicles are empty with a few ova and aggregations of hemocytes. (F) Male; Spawning stage 2. Sperm is being released from the gonad in the seawater. Scale bars 300 µm.

licles, ova filled the lumina (Fig. 2C) and were angular in shape until spawning at which time they became more rounded. In ripe male follicles, different stages of spermatogenesis could be observed. Germinal epithelium lining the follicles was overlaid by spermatogonia and spermatocytes with numerous meiotic and mitotic figures present. Mature sperm with pink staining flagella filled the middle of follicles (Fig. 2D). At the end of spawning, follicles, with some ripe gametes present, began to shrink and were replaced by connective tissue (Fig. 2E, F).

Gonad indices of the mussels are illustrated in Figure 3. Mussels had already begun gametogenesis in February when sampling started. The major spawning peak was in May followed by a minor one in August. Percentages of developing, spawning, and spent mussels are shown in Figure 4. Spawning specimens were present from April until January with a sharp decline in July, when half of the mussels were again developing and preparing for the fall spawning. Most of the mussels showed some gonad development throughout the entire year. However, in December 50% of specimens examined were resting with no developing gametes present in their mantles, and sexes could not be distinguished (see Fig. 4).

Pathologic Observations

Pathologic observations on histologic sections are listed in Table 2. Four different types of infectious organisms were observed. Intracellular prokaryotes (*Chlamydia*, *Rickettsia* or *Mycoplasma*) were present in digestive epithelial cells of one specimen. These organisms formed basophilic, finely granular inclusions. Ciliates (*Ancistrum mytili*) were observed on the gills of some mussels. Ciliates were pear shaped with a macro- and micro-nucleus and were anchored between gill epithelial cells. A small percentage (5.8%) of the mussels had microsporidian infections, *Steinhausii mytilovum* (Mussel Egg Disease). Parasites formed vacuoles inside the cytoplasm or nuclei of ova where large numbers of microsporida were observed with their characteristic polar vacuoles (Fig. 5). Also, immature cysts with less defined, developing parasites were present. There was an accumulation of granular hemocytes in the follicles containing infected ova. The pres-

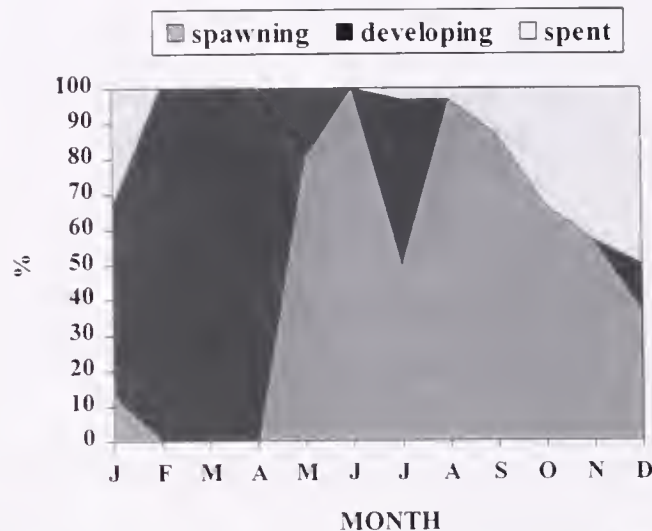


Figure 4. Percentage of spent, spawning, and developing mussels (*Mytilus edulis*) during 1 year in a long line culture in Long Island Sound, Connecticut.

ence of *S. mytilovum* was first detected in June (23.3%), after which they were present at prevalences ranging from 0% to 13.3% (Fig. 6). Although *S. mytilovum* occurred at high prevalence, the proportion of infected ova versus uninfected ova was generally low. In some specimens, several infected ova were present in the same follicle, and several vacuoles were present in a single ovum (Fig. 5).

The October mussel samples exhibited infections by the digenetic trematode, *Proctoeces maculatus*. The high prevalence of

TABLE 2.

A list of infectious organisms, pathologic responses and their average prevalences based on histopathological observations over the course of 1 year in mussels (*Mytilus edulis*, n = 360) from a longline culture in Long Island Sound, Connecticut.

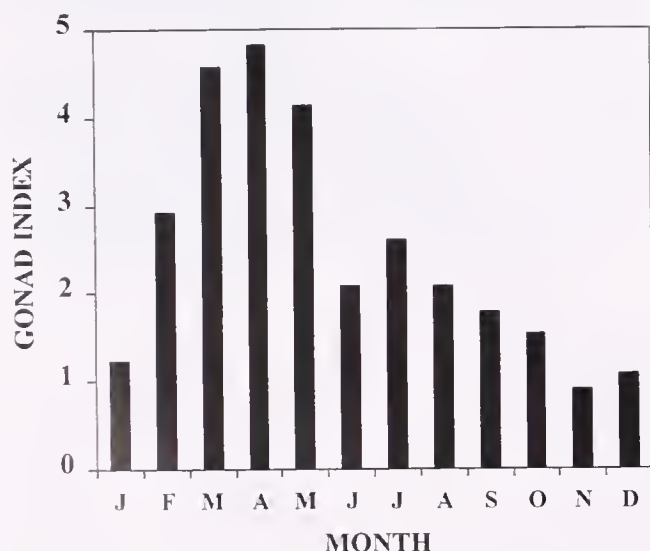


Figure 3. Gonad indices of mussels (*Mytilus edulis*) over the course of 1 year in a long line culture in Long Island Sound, Connecticut. Indices can vary from zero, when no sexual activity is noted, to five, when all the individuals are mature.

Histopathological Observations	Prevalence %
Infectious organisms	
Prokaryotic inclusions in digestive cells (<i>Chlamydia</i> / <i>Rickettsia</i> / <i>Mycoplasma</i>)	0.3
<i>Ancistrum mytili</i> on the gills	3.1
<i>Steinhausii mytilovum</i> in the ova	5.8
<i>Proctoeces maculatus</i>	20.8
Pathologic responses	
Acute inflammation:	
Multifocal aggregation of hemocytes in the byssus gland	0.3
Focal aggregation of hemocytes in the digestive diverticulum	0.3
Abscesses in the digestive diverticula or the kidney	1.1
Chronic inflammation:	
Pearl formation	0.8
Degenerative responses:	
Degeneration of digestive tubules with collagen production	0.3
Abnormal byssal thread formation	0.3
Kidney concretions	3.3
Trauma:	
Hemorrhage in the stomach	0.8

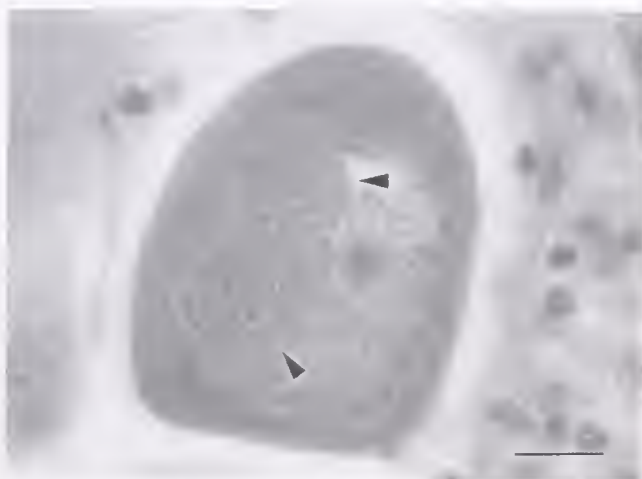


Figure 5. *Steinhausii mytilovum* (Microsporidia) in an ovum of a mussel (*Mytilus edulis*). Hematoxylin-eosin stained section. There are 2 vacuoles in the nucleus containing the parasites (arrows). Polar vacuoles of the microsporidia can be seen. Scale bar 20 μ m.

infection (67%) remained until the end of sampling (Fig. 6). Trematodes invaded the gonadal follicles and different stages of maturation, from sporocysts containing germ balls and cercaria to metacercaria, could be observed (Fig. 7A). More mature forms were present in the foot musculature and the pericardial chamber. Occasionally, large abscesses were formed, harboring adult forms of the parasites. These stages of the trematodes had oral and ventral suckers (Fig. 7B) and occasionally the digestive tract appeared to be filled with host hemocytes (Fig. 7C). Sperm and ovaries, as well as ova with developing miracidia within them, were present in several trematodes. Massive hemocytic response surrounded some of the worms. Parasites in different stages of degradation, due to the response of host hemocytes, were seen towards the end of the sampling period. However, in most cases the parasites did not

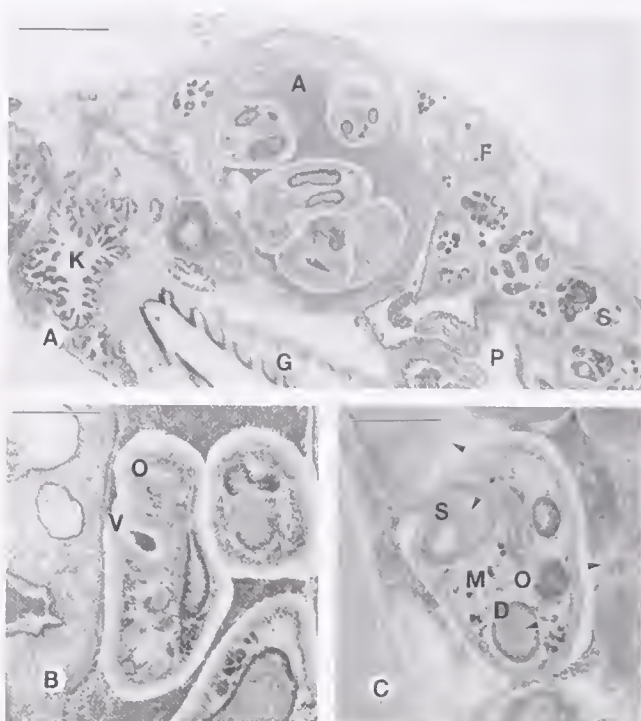


Figure 7. *Proctoeces maculatus* (Trematode) in mussels (*Mytilus edulis*). (A) An abscess in the mantle with 5 almost adult trematodes. The abscess is filled with hemocytes. Different stages of immature trematodes fill the follicles with no apparent host response. A = abscess, K = kidney, F = follicles containing sporocysts, G = gills, P = plicate membranes, S = sporocysts. Scale bar 500 μ m. (B) *Proctoeces maculatus* with oral (O) and ventral (V) suckers. Scale bar 250 μ m. (C) *Proctoeces maculatus* in the pericardial cavity surrounded by hemocytes (arrows). Features of this adult trematode include: sucker (S), digestive tract (D), an ovary (O) with mature ova, as well as developing miracidia (M). Scale bar 250 μ m.

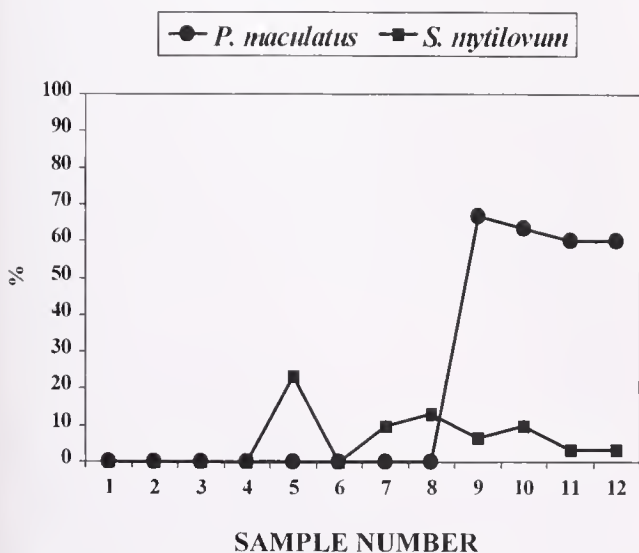


Figure 6. Percentages of mussels (*Mytilus edulis*) infected with *Proctoeces maculatus* (Trematodae) and *Steinhausii mytilovum* (Microsporidia) during 1 year. Monthly sampling of the same cohort of mussels started in February 2002 (1 on x-axis) when the stock was 6 months of age, and ended in January 2003 (12 on x-axis).

evoke any host response and appeared to have completed their life cycle inside the mussels. Trematodes invaded the follicles, foot, and pericardial cavity, as well as the digestive gland and kidneys; some of the infested mussels were merely a mass of trematodes in different stages of development. Surprisingly, heavily infested mussels appeared healthy even when the tissues were almost entirely displaced by the parasite mass.

The pathologic responses in the mussels included multifocal aggregations of hemocytes in the byssus gland in one specimen, focal aggregation of hemocytes in the digestive diverticulum in one specimen, and abscesses in the digestive diverticulum or the kidney in four specimens (Table 2). Three specimens had pearls in the mantle epithelium. One specimen had degeneration of digestive tubules with deposition of collagen in the surrounding connective tissue. This lesion was surrounded by hemocyte infiltration. Abnormal byssus formation with threads forming concentric structures inside the septal area of the byssus gland and not in the foot groove, as usually occurs, was present in one specimen. Kidney concretions, located inside kidney tubules with varied sizes and shapes, were observed in 12 specimens. There were also hemocyte aggregations in the stomachs of three specimens.

No significant mortalities were observed during the experiment. The meat quality remained high until the end of the summer, after which the mussels were thin and watery with visible trematode abscesses in the mantles. Concurrently, the mussels started to

slide down the ropes due to their collective weight on the dropper lines.

DISCUSSION

Blue mussels occur on the East Coast of North America from the Canadian Maritimes to Cape Hatteras, North Carolina (Gosner 1978, Koehn et al. 1984). Mussels are successfully cultured on the East Coast of the US in Maine and Prince Edward Island (PEI) in Canada. There is no commercial mussel culture south of Long Island Sound. High temperatures may have concurrent negative and positive effects on the culture, therefore, evaluating the biologic potential of mussel longline culture in LIS should be cognizant of these factors.

On the East Coast of North America there are 2 species of blue mussels, *Mytilus edulis* and *M. trossulus*. These species do not have reliable morphologic characteristics and confusion about their taxonomy prevails throughout blue mussel research. Blue mussels in northern Canada and Alaska are mainly *M. trossulus*, but in Newfoundland and Nova Scotia, Canada *M. trossulus* overlaps with *M. edulis* (Penney & Hart 1999). In Maine there is a transition zone where *M. trossulus* dominates at some sites in the northeast whereas *M. edulis* is the prominent species in central Maine (Rawson et al. 2001). Several commercial mussel farms operate successfully with a mixed seed stock of *M. edulis* and *M. trossulus* (Mallet & Carver 1995). *M. trossulus* is considered to have an economic disadvantage compared with *M. edulis* in regard to tissue weight and shell height. Mallet and Carver (1995) estimated that the economic value of *M. edulis* was 1.7 times higher than that of *M. trossulus*. The only mussel genotype present in LIS is *M. edulis* (McDonald et al. 1991). This is an obvious benefit for the potential of a LIS mussel longline culture.

Mussels had an exceptional growth rate on the longline reaching market size (60 mm) during the first 6 months after seed set (Table 1). There was no significant additional growth during their second summer (Fig. 8) thus demonstrating that the population had

reached their full size during the first growing season. Growth rate is an important criterion in assessing the potential for mussel culture in a given area. Depending on geographic latitudes and environmental factors (temperature, light, salinity, primary productivity, currents, and tides), growth of mussels varies. In Iceland, mussels reach market size (50 mm) in 24 mo after settlement (Thorarinsdóttir 1996). In Galicia, Spain, mussels (*Mytilus galloprovincialis*) generally reach commercial size (80–90 mm) in 12–18 mo. In France, *M. edulis* reach commercial size (40–50 mm) in 24 mo, and in the Netherlands commercial size (72 mm, bottom culture) is reached in 36 mo (Chew & King 2000). Langan and Horton (2002) reported a 13-mo production cycle to 55 mm in their New Hampshire deep-water longline. In PEI the production cycle varies from 17–28 mo (McDonald et al. 2002).

No significant mortality was detected in the mussels during the experiment. However, massive mussel beachings on the shores of LIS were observed during the past summers (Sunila 2001). Mussel beaching occurred in the area during August and September of this experiment. This phenomenon affects intertidal mussels during unusually hot summers in LIS. During emersion mussels gape slightly for air breathing, which exposes the animals to desiccation and possible death (De Zwaan 1977). Mussels react to environmental stress, such as high temperature, by cutting their byssi off and trying to sink. During hot summer days emersed mussels jettison their byssi, but because of gas bubbles trapped between their shells they are unable to sink and instead float. Animals are finally washed on shore in piles that may reach a meter high (Sunila 2001). Beached mussels are live and based on histologic observations in good health, but without byssi (Sunila, pers. obs.).

Summer mortality is a well-published and frequent event in cultured mussels (e.g., Myrand & Gaudreault 1995, Incze et al. 1980, Emmett et al. 1987). According to Myrand and Gaudreault (1995) as much as 80% of the population can be affected in PEI. The phenomenon usually affects mussels during their second growing season. There can be significant differences in the temperature tolerance of mussels originating only kilometers apart. Susceptibility to summer mortality appears to be due more to the genetic origin of the mussels than to culture site. Incze et al. (1980) reported decreased growth rate of mussels in an experimental raft culture in Maine when water temperature exceeded 20°C. Thompson and Newell (1985) compared mussels from Newfoundland, Canada and mussels from LIS (Stony Brook, NY) and demonstrated more severe stress (measured as depression of the clearance rate) at 25°C in the Newfoundland mussels than the LIS mussels. The Newfoundland mussels also had a considerably higher mortality rate (46%) than the LIS mussels (10%) at 25°C. They concluded that the mortality patterns and physiologic responses suggested that mussels from southern, warmer waters are better adapted to high temperatures than mussels from northern, cooler waters.

Along with rising seawater temperature, mussels acquired *Protoeces maculatus* (Looss 1901), Fellodistomidae, infection (Table 2 and Fig. 6). At the same time mussels started to slide down the ropes, their meats became watery and thin and parasite abscesses were observed on the mantles by the naked eye. In the past, this condition has been referred to as "orange sickness" due to the characteristic orange color of the parasite. The first to observe *P. maculatus* in LIS mussels was Uzzmann (1953), who named them *Cercaria milfordensis*. He reported a prevalence of 4.3% from Mill Neck, Long Island, New York and prevalences of 6.6% and 7.7% in subsequent years in a pooled sample from Mil-

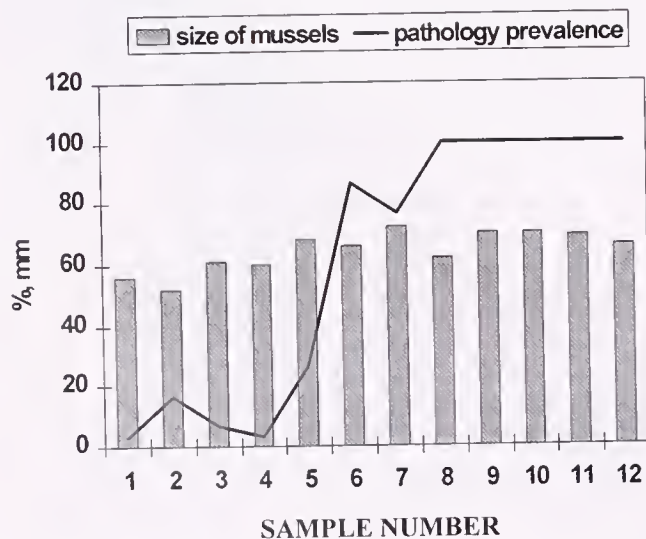


Figure 8. Size of mussels (*Mytilus edulis*) in a long line culture in Long Island Sound, Connecticut, starting 6 months after the seed settled. Solid line depicts the pathology prevalence, which includes all parasites and pathologic responses in the mussels as well as fouling organisms. Time scale on the x-axis as in Figure 6, y = axis indicates the size of the mussels in mm and the pathology prevalence as %.

ford and Bridgeport, CT. Later, Stunkard and Uzmann (1959) sampled *Mytilus edulis* from Connecticut and Massachusetts and observed several life stages of these trematodes, from unencysted metacercariae to gravid adults, in the mussels. They concluded that *C. milfordensis* is the larval stage of *P. maculatus*. *Cercaria tenuis* and *C. brachidontis* also were conspecific with *C. milfordensis* and the cercarial stage of *P. maculatus*. This species may be able to complete its entire life cycle in mussels and a vertebrate final host is not needed. However, in more southern locations tropical and subtropical shallow water bottom feeding marine fish belonging to several different families are reported to harbor adult *Proctoeces* in their hindguts (Wardle 1980). The ability to accomplish their life cycle within the mussel host may represent a mechanism for *P. maculatus* to extend its limits of distribution into temperate waters without dependence on its usual tropical fish hosts (Lang & Dennis 1976).

Data about the economic importance of this cosmopolitan parasite to the mussel industry are controversial. Robledo et al. (1994b) reported a low prevalence (less than 1/1000) of *P. maculatus* in *M. galloprovincialis* in Spain and concluded that although the parasite caused severe lesions, the low prevalence did not make it a real threat to the mussel industry. This view was supported by Villalba et al. (1997) who reported average prevalences of 0.7% from different locations in Spain, and Canzonier (1972) who reported prevalences of 4% from Ria de Arosa, Spain and 4% in Laguna Veneta, Italy. The parasite was later associated with extensive mortalities of cultured mussels in Laguna Veneta (Munford et al. 1981). However, other etiologic agents as putative causes of the observed mortality were not excluded in the study. In this study, there was no mortality associated with the infection, and the presence of disintegrating parasites surrounded by a strong host response at the end of the year suggested that the mussels were able to suppress the parasites and modulate their life cycle. However, the exceptionally high prevalences observed in this study and Uzmann's (1953) early observations suggest that the mussel longline was deployed right in the midst of an area with high infection potential.

P. maculatus elicited a strong hemocyte response in the host mussel (see Fig. 7). This differs from most mollusk-trematode infections when the host usually doesn't react to healthy parasites. Tripp and Turner (1978) described adult *P. maculatus* in the pericardial cavity of mussels from New Jersey. They claimed that the trematodes actively ingested hemocytes and actually "grazed" the mussel tissues. Besides eliciting hemocyte response, *P. maculatus* affects reproduction of the mussels. Feng (1988) studied infected mussels from Ram Island Reef, CT. He reported 33% mean prevalence of infection. In moderate to heavy infections, normal gametogenesis was either impaired or totally absent in 6% to 11% of the infected mussels.

The prevalence of *P. maculatus* in this study showed seasonality with the trematode absent until August leading to epizootic prevalences in fall and early winter (see Fig. 6). A similar trend was described by Lang and Dennis (1976) in New Jersey and Tripp and Turner (1978) in Delaware. Uzmann (1953) noted that although mussels were extensively studied in the higher latitudes for years, the presence of *P. maculatus* had not been reported. Based on the results of this study, those of Lang and Dennis (1976), and Tripp and Turner (1978), we conclude that *P. maculatus* appears at high seasonal prevalences in the mussels close to the southern border of their distribution and could likely affect the potential of mussel culture in this region.

Mussels also acquired infestation with pea crabs, *Pinnotheres maculatus* (see Table 1). Pea crabs were absent or occurred at low prevalences (3.3%) from February until November when their prevalence reached 20% and peaked to 60% in January. Pea crabs impacted a large portion of the mantle cavity causing deformed gills and a weak and watery visceral mass in infested specimens. Seasonal prevalences of the pea crabs followed what was previously reported about the life cycle of pea crabs in the area (Kelly 1988). The larval stages infest the mussels in mid-September following initial planktonic stages. After several molts, the adult pea crabs leave the host to mate in a copulatory swarming in open water by mid-October, and inseminated females reinfest the host for the winter. Few males are found in mussels following the mating. Males live 1 year or less, and are significantly smaller, while females live 3 to 4 years (Kelly 1988, Pearce 1964).

Proctoeces maculatus infection and *Pinnotheres maculatus* infestation clearly pose a threat to the health of mussels and subsequently to the potential of commercial culture in LIS. Both parasites lowered the product quality and would likely affect consumer perception. In addition, human consumption of trematode-infested mollusks may be hazardous due to the accumulation of toxic metabolites (butyric and other short-chain fatty acids) resulting from degeneration of the hosts' neutral fats by parasite-secreted enzymes (Bower & Figueras 1989). A third parasite of regulatory significance was *Steinhausii mytilovum* (Mussel Egg Disease). This can be classified as a parasite that can damage the mussel, but is unlikely to be lethal to the host (Villalba et al. 1997). Microscopical details of the parasite were presented by Sprague (1965) and Sindermann (1990) assigned the protozoan to the phylum Microspora. The condition, naturally, affects only females. The parasite was first observed in maturing ova in the beginning of gametogenesis (Fig. 5). Prevalence decreased in June after the first spawning, but increased again with the second spawning peak (Fig. 6). The infection is believed to affect the fecundity of mussels (Villalba et al. 1997), but this view was disputed by Robledo et al. (1994b) who stated that the low number of infected ova would be an insignificant factor because of the large number of ova produced by the animals.

There were several other parasites and pathologic conditions present that are considered of no commercial importance (Table 2). Prokaryotic inclusions were observed inside digestive epithelial cells of one specimen (0.3%, Table 2). These can be caused by Rickettsia, Chlamydia, or Mycoplasma, which are obligate intracellular organisms indistinguishable from each other under light microscopy. While they cause serious diseases in humans and domestic animals, micro-organisms from these three groups are considered ubiquitous and of low pathogenicity to marine bivalves (Harshbarger et al. 1977, Lauckner 1983). Finally, there was a low prevalence (3.1%, Table 2) of the ciliate *Ancistrum mytili* on the gills of the mussels. However, this species is considered ubiquitous and not of regulatory significance (Moret et al. 1999).

Three of the mussels contained pearls (Table 2), a condition generally attributed to the trematode metacercaria in the family *Gymnophallidae*. The number of pearls in the mussels in Maine increased with age from zero in 2-year class animals up to an average of 43 pearls/mussel in 8-year-old mussels (Lutz & Hidu 1978). Pearl formation may seriously affect marketability of these animals, but because of the fast growth rate of the mussels, pearl formation would not have time to reach prevalences that would affect a LIS suspended culture.

Abnormal byssal thread formation was observed in one speci-

men in the present experiment. The condition was previously reported by Sunila (1987). In the affected mussel, byssus threads formed inside the hyssus glands as massive concentric structures may impair the attachment of the mussel. This condition is insignificant because of the low prevalence.

Kidney concretions were also observed in some mussels (Table 2). The concretions are formed principally of amorphous calcium phosphate (Doyle et al. 1978) and are larger and more numerous in quahogs (*Mercenaria mercenaria*) from polluted areas than in kidneys of quahogs obtained from more pristine areas (Rheinberger et al. 1979). Presence of kidney stones in LIS mussels is not surprising considering the anthropogenic stress in the area. Kidney stones in mussels were previously reported by Sunila (1987) who observed higher prevalences in sampling locations closer to an iron and steel factory compared with animals sampled from more pristine bottoms in the Baltic Sea.

While evaluating biologic potential for mussel longlines in LIS, two opposite forces clearly came together in the area. The first, a positive factor, was the exceptional growth rate that occurred compared with rates previously published for commercial mussel operations. The second, a negative factor, was the epizootic prevalence of *Proctoecces maculatus*, which is a species usually characteristic of tropical and temperate regions. Because of its high prevalence in sampled animals *P. maculatus* could be considered an economically important parasite of mussel culture in the area. Figure 8 demonstrates the size of mussels and pathology prevalences for each sampling month. The presence of fouling organisms was also included in the pathology prevalence data. There was a 5-month "window of opportunity" for mussel culture between February and June, during which mussels were already of marketable size, free of fouling organisms and parasites and had good quality meats. Based on histologic sections, mussels were also healthy in July and August, but at that time they were extensively fouled with *Crepidula fornicata*. Providing a marketable product during those months would require the use of cleaners and debysers. No investments were made to import such commercially available equipment to the area during the present, experimental stage of mussel culture. However, the fast growth, good quality of meat and the unfouled shells between February to June suggest the potential for a seasonal product.

The reproduction data of this study indicates that seed lines in the Milford area should be deployed in June. Although spawning specimens dominated in June (Fig. 4), the gonad index had already decreased, indicating that the spawning peak was in May (Fig. 3). When predicting the right timing for future seed line deployments it must be kept in mind that mussel spawning and setting times differ significantly in different areas in LIS as well as interannually (Fell & Balsamo 1985, Brousseau 1983, Newell et al. 1982). The eastern end of LIS is more oceanic and differs significantly from the western, estuarine end of LIS. Accordingly, in the eastern end of LIS, mussel spawning followed the pattern of more oceanic mussel populations (Fell & Balsamo 1985). This is in accordance with Newell et al. (1982) who observed much later spawning in Shinnecock, NY (more oceanic south shore of Long Island, Atlantic Ocean) than in Stony Brook, NY (more estuarine north shore of Long Island, LIS). Therefore, providing seed for commercial operations would require the deployment of multiple seed lines, timed differently in different parts of the sound.

Apart from the biologic factors, expansion of mussel longline culture in LIS could meet serious regulatory obstacles. LIS, "The Urban Sea," is a heavily populated and highly prized body of water, and any attempt to use the water column would conflict with the needs of different user groups, such as recreational boating and fishing in inshore areas and commercial ship traffic in deeper water. However, small-scale culture of mussels could provide supplemental income to shellfishermen seeking to diversify their business.

ACKNOWLEDGMENTS

The authors thank Mr. Walter Canzonier from Port Norris, New Jersey and Mr. James Winstead from EPA, Florida for their constructive and extensive reviews of the draft manuscript. Mr. Winstead also helped in the diagnosis of *Proctoecces maculatus* trematode. Dr. Lillemor Svärth from Tjärnö Marine Biological Laboratory, Sweden is appreciated for her input in the early stages of the manuscript. Shannon Kelly is acknowledged for detailed editing of the manuscript. This work was supported in part by Connecticut Sea Grant, Development Project M/DP-1 (2001).

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FIRST OCCURRENCE OF THE NONINDIGENOUS GREEN MUSSEL, *PERNA VIRIDIS* (LINNAEUS, 1758) IN COASTAL GEORGIA, UNITED STATES

ALAN J. POWER,*¹ RANDAL L. WALKER,¹ KAREN PAYNE² AND DORSET HURLEY³

¹Shellfish Research Laboratory, University of Georgia Marine Extension Service, 20 Ocean Science Circle, Savannah, Georgia 31411; ²School of Marine Programs, University of Georgia Marine Extension Service, 220 Marine Sciences Building, Athens, Georgia 30602; ³Sapelo Island National Estuarine Research Reserve, GA Department of Natural Resources, P.O. Box 15, Sapelo Island, Georgia 31327

ABSTRACT Since being accidentally introduced into Tampa Bay in the Gulf of Mexico in 1999, the green mussel (*Perna viridis*), a native to the Indo-Pacific region, has proliferated and dispersed southwards along peninsular Florida. During 2002 another introduction of *P. viridis* occurred on the northeast coast of Florida and larval dispersal has also carried the species northward in Atlantic coastal waters. In anticipation of their arrival in Georgia, a campaign was initiated to alert recreational and commercial water users to report all sightings. Observations of living and dead mussels trickled in during 2003 from all over coastal Georgia, confirming the presence of the mussel here for the first time and representing the expansion of the range of this invasive species to its most northerly location in the United States.

KEY WORDS: green mussel, *Perna viridis*, Georgia, invasive

The green mussel, *Perna viridis* (Linnaeus, 1758) is a commonly found mussel of the family Mytilidae in its native tropical waters of the Indo-Pacific region of Asia (Siddall 1980). There the species form the basis for an important aquaculture industry (Qasim et al. 1977, Sivalingam 1977, Sreenivasan et al. 1989, Zhang et al. 1997, Chalermwat et al. 2003). The species is dioecious, sexually matures at 2 to 3 mo of age, lives for about 3 y, and it typically attaches itself to hard structures with byssal threads in densities of up to 35,200 individuals/m² (Huang et al. 1985, Lee 1985, Lee 1988, <http://www.fcsc.usgs.gov>). Green mussels occur in coastal waters (<10 m), in salinities between 16 and 33 ppt, at temperatures between 10°C and 35°C (optimal: 27 to 33 ppt, 26°C to 32°C), and exhibit a wide tolerance for turbidity concentrations and pollution (Sivalingam 1977, Sundaram & Shafee 1989, Lee 1986, Tan 1997, Masilamoni et al. 1997, Benson et al. 2001). In parts of their native range, they exhibit rapid growth rates of between 6 and 10 mm/month (Lee 1985, 1986, Walter 1982). These biologic attributes facilitate opportunistic colonization of new areas.

In 1990, the mussel appeared off Trinidad in the Caribbean for the first time, then in 1993 off Venezuela, later in Jamaica, and subsequently in Tampa Bay, Florida in 1999 (Agard et al. 1992, Rylander et al. 1996, Ingrao et al. 2001, Benson et al. 2001). Ballast water releases are believed to have been the most likely route for introductions. In Tampa Bay, this species has fouled bridges, piers, and buoys, and it has even displaced oyster (*Crassostrea virginica*) reefs (Baker & Benson 2002). In Tampa Bay, juveniles have been recorded at densities of 9,000 to 12,000/m², and adults at 1,000 to 4,000/m²; growth rates are rapid, reaching 4–5 mm/wk; and individuals are reproductively capable at between 1 and 2 mo of age (Jonathan Fajans, University of Florida, Department of Fisheries & Aquatic Sciences, pers. comm.). From Tampa, currents have dispersed larvae south along the Gulf Coast to Boca Grande outside of Charlotte Harbor (Benson et al. 2001).

In 2002, reports of the green mussel surfaced on the northeast coast of Florida in the St. Augustine area (Dr. Richard Gleeson, Guana Tolomato Matanzas National Estuarine Research Reserve).

Because the mussel has not yet arrived in the Florida Keys, it is unlikely that the occurrence of the green mussel in Georgia resulted from larval dispersal around the peninsula. This may represent an entirely new introduction, however it is more likely to have resulted from boats and equipment being transferred between coasts without adequate cleaning of attached organisms and draining of bilge water. During early 2003, currents carried the mussel as far north as Jacksonville Beach, Florida and fears arose that it would soon appear in Georgia.

The University of Georgia Marine Extension Service (UGA MAREX) in collaboration with the University of North Carolina at Wilmington are currently conducting a Sea-Grant-funded biologic survey of the major shipping ports in the southeastern United States (Jacksonville, Florida; Savannah, Georgia; Charleston, South Carolina; and Wilmington, North Carolina) for invasive species. The Sapelo Island National Estuarine Research Reserve (SINERR) is also investigating invasives in the Sapelo Island area of coastal Georgia as part of a national monitoring program. In anticipation of the green mussels' arrival, UGA MAREX and SINERR prepared an informative flyer, which was distributed to commercial fishermen, and posted it at marinas and public boat ramps throughout coastal Georgia. The species appearance and biology was described, a photo was included, and anyone finding one of these mussels was urged to collect the specimen and file a report.

The first report in Georgia came in early 2003, from biologists in the Department of Natural Resources (DNR), who observed many green mussels inhabiting artificial reef G, approximately 30 miles offshore from Brunswick (Fig. 1). Green mussels were again observed there during the summer months. Later in the fall, we began to receive reports from throughout the coastal area of Georgia between Savannah and Brunswick (Plantation Creek, Mackay River, Fredrica River, Savannah River, Skidaway River, and Tybee Beach; see Fig. 1). Mussels were collected from the surface to approximately 4.5 m in depth. Mussels were found subtidally on old abandoned crab traps, crab trap floats, rope, boat fenders, and also in the lower intertidal zone on beach jetties. With the exception of the jetties, all other reports were single specimens. The largest living specimen recorded was 70 mm in length, 35 mm in height, and 23 mm in width. The average shell length of

*Corresponding author. E-mail: alanpowr@uga.edu

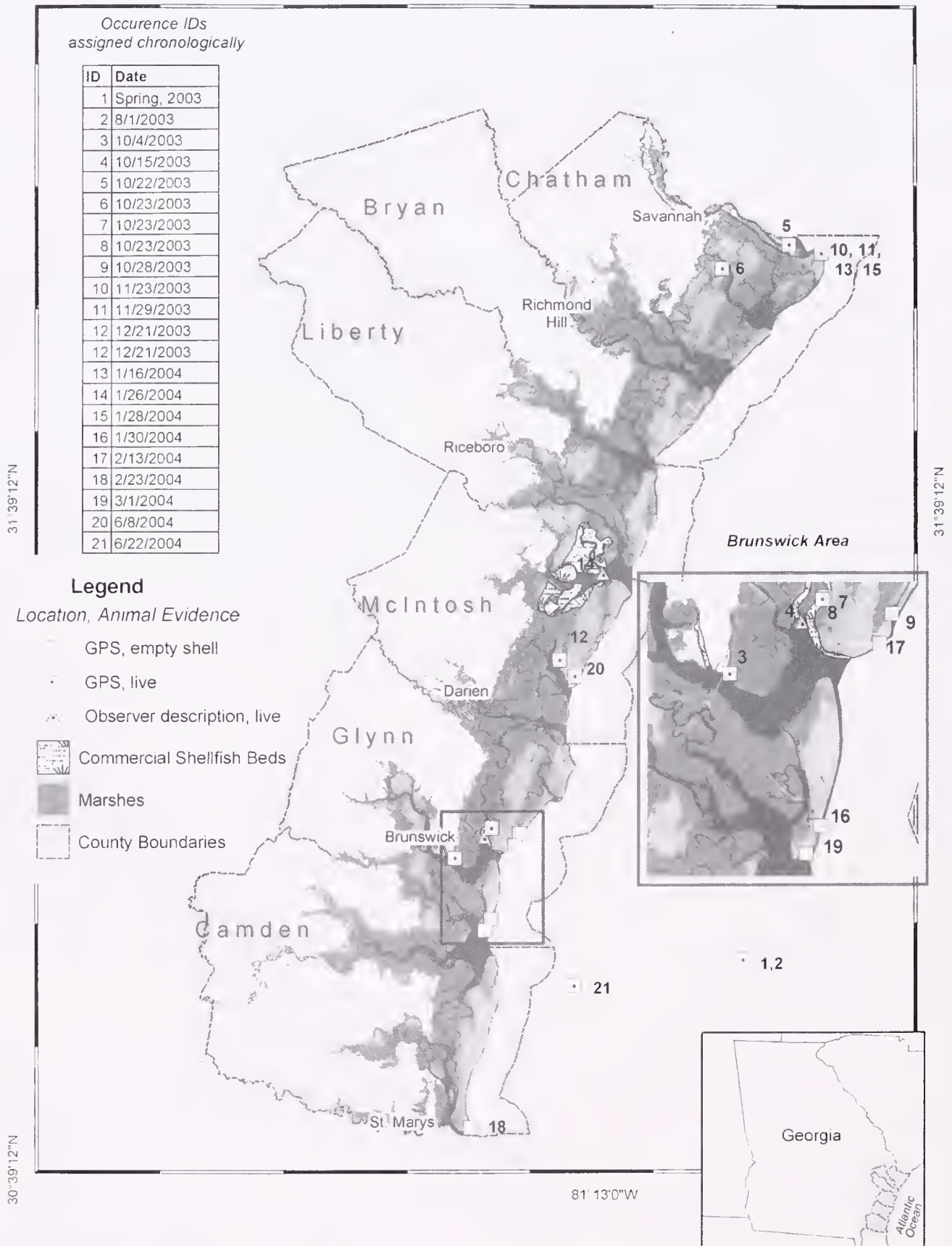


Figure 1. Reported occurrences of the green mussel, *Perna viridis* throughout the coastal counties of Georgia (GA). Observations are numbered chronologically and each specimen is identified as living or dead, and located by GPS or observer descriptions. Because many occurrences were reported from Brunswick, an enlarged insert of this area is also included.

all mussels found during the fall ($N = 18$) was 34.44 mm (± 2.98 S.E.).

During winter 2004, all intertidal individuals on the jetties died. A greater tidal range exists here than in Tampa, and possibly the longer periods of exposure to low air temperatures will limit the mussels' survival in the intertidal zone. This is beneficial to Georgia's oyster reefs, which are primarily intertidal (Harris 1980). Green mussel shells were washed ashore coastwide over the winter (Sea Island, Tybee Island, Jekyll Island, St. Simons Island, and Cumberland Island; see Fig. 1). The largest shell washed ashore measured 95 mm in length.

Only one resident live mussel has been found in inshore waters in 2004; it was pulled from an abandoned crab trap in Sapelo Sound during January (see Fig. 1). It is likely that others have survived the winter in the subtidal zone. On June 8, 2004 a single specimen was found in Dean Creek near Sapelo Island, attached to a floating piece of wood (see Fig. 1). Six live mussels were also collected by the DNR on June 22, 2004 from buoy markings at Georgia Artificial Reef "A", which are 7 nautical miles east of Little Cumberland Island (see Fig. 1). These individuals ranged in shell length from 28.8 to 73.4 mm (mean \pm SE = 50.0 ± 7.9). We may see newly recruited mussels occurring in inshore waters during the warm summer and fall months, because the lower temperature threshold for gametogenesis of 24°C (Lee 1988) is typically attained during the month of May in Georgia.

These observations represent the current most northerly reported occurrence of the species in the United States. None have yet been recorded from South Carolina (David Knott, South Carolina Department of Natural Resources, Southeastern Regional Taxonomic Center, pers. comm.). However, established breeding populations in Georgia in combination with mild winters would make this expansion a likely scenario. In Japan, where the species has also been introduced, populations have managed to survive the severest winters in certain areas due to warm water discharges from several factories (Umemori & Horikoshi 1991).

An interesting side observation of the present survey was the rare documentation of 2 *Mytilus edulis* (L.) individuals in October 2003, one from the Brunswick area and another from Sapelo (identified by Dr. Paula Mikkelsen, American Museum of Natural History). This common bivalve of the northeastern United States region typically does not occur as far south as Georgia.

The zebra mussel, *Dreissena polymorpha* (Pallas 1771), a native of Eastern Europe, is an infamous freshwater ballast invader that occurs in over 40% of America's inland waterways. Close to \$1 billion has been spent on controlling the species in this country over the last 15 years. A marine counterpart to the zebra mussel in the southeastern United States could be the green mussel. Witnessing the economic and environmental problems experienced in the Tampa Bay region on the Gulf Coast of Florida since the introduction of the green mussel there a few years ago, has led to considerable concern in the coastal community of Georgia. UGA and SINERR have recently initiated a specific green mussel monitoring survey in which devices that offer a variety of settling surfaces (Hester Dendy fouling plates, rope, artificial seaweed, and pvc sheets) have been deployed subtidally in various salinities throughout coastal Georgia. Quarterly sampling will provide an assessment of the distribution and abundance of the species here in Georgia and will provide information about its ecological and environmental tolerances for eventual prediction of the geographic and ecological impacts that could occur due to this organism.

ACKNOWLEDGMENTS

The University of Georgia Marine Extension Service, Sapelo Island National Estuarine Research Reserve and NOAA's National Sea Grant Aquatic Nuisance Species Program provided funding for this work. Thanks to Dr. Amy Benson (United States Geological Survey), Mr. Jonathan Fajans (University of Florida), Dr. Harry Lee (Jacksonville Shell Club), and Dr. Richard Gleeson (GTMNERR) for providing information on the Florida green mussel invasion. Thanks to Dr. Marc Frisher (Skidaway Institute of Oceanography) and Dr. Paula Mikkelsen (American Museum of Natural History) for assisting with the positive identification of specimens. Finally, thanks to all those who have participated in these surveys: Mr. Dwight Vernado, Mr. Henry Ansley, Mr. Dominic Guadagnoli, Mr. Brooks Good, Mr. Gabe Gaddis (Georgia Department of Natural Resources), Ms. Ellie Covington, Ms. Marcy Mitchell, Ms. Mary Sweeney-Reeves, Mr. James Nichols, and Dr. Mare Timmons (University of Georgia Marine Extension Service), Dr. Dale Bishop (University of Georgia School of Marine Programs), the Jekyll and Tybee Island 4-H Centers, the Tybee Island Marine Science Center, and members of the public.

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EVALUATION OF THE NEUTRAL RED RETENTION ASSAY AS A STRESS RESPONSE INDICATOR IN CULTIVATED MUSSELS (*MYTILUS* SPP.) IN RELATION TO SEASONAL AND ENVIRONMENTAL CONDITIONS

JOANNE M. HARDING,¹ CYR COUTURIER,¹ G. JAY PARSONS^{1*} AND NEIL W. ROSS²

¹Fisheries and Marine Institute, Memorial University of Newfoundland, P.O. Box 4920, St. John's, Newfoundland and Labrador, Canada A1C 5R3; ²National Research Council, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, Nova Scotia, Canada B3H 3Z1

ABSTRACT The neutral red assay (NRA) was evaluated as an indicator of stress response in mussels that were held under various culture situations. The NRA measures retention time of neutral red dye in the hemocyte organelle, the lysosome, which can be correlated to the condition of a mussel under stressful circumstances. Shelf life and standard meat yield also provide an indication of mussel condition. The objectives of this study are to compare and evaluate mussel stress response in relation to: (1) seasonal and environmental changes and (2) postharvest handling. Neutral red retention (NRR) levels and shelf life were reduced in late summer mussels (postspawning) compared with early summer mussels (prespawning), and increased in autumn/early winter mussels, indicating a seasonal pattern of stress response associated with reproduction. Harvested mussels exhibited a decrease in NRR during extended air exposure, especially when held at air temperatures above and below air temperatures comparable to ambient water temperatures. The results demonstrated that NRA was a useful, sensitive index of physiological stress response in mussels subjected to various culture practices and conditions. The implication of this work for mussel growers is that reduced air exposure following harvest and reduced handling during certain seasons will result in less stressed mussels and hence a better quality product.

KEY WORDS: mussel culture, neutral red assay, stress response

INTRODUCTION

The blue mussel (*Mytilus* spp.) culture industry in Canada has been on a rapid increase since the mid 1980s. In Newfoundland, with the onset of various programs such as the spatfall monitoring program, environmental and biological monitoring program, and product quality programs, the overall quality and quantity of blue mussels continues to improve (Clemens et al. 1999, Macneill et al. 1999). Because the market demand continues to rise for the Atlantic Canada mussel industry, a better understanding of how various production parameters (i.e., environmental and handling conditions) affect mussels need to be examined and evaluated to determine when or how conditions may become detrimental and reduce the quality of the product.

During the process of culturing mussels, there are several seasonal and short-term environmental factors that the mussels are exposed to while in the growout phase, including changes in water temperature, salinity, and food availability. Stress can be defined as any environmental stimulus that disturbs normal function in an organism, and that any reaction in the organism to this stress is the stress response (Bayne 1985). Under some circumstances conditions during the culture of mussels can have a negative impact on the mussels and may induce a stress response. Stress response in animals is dynamic, and results in the alteration of functional properties that can be quantified with the environmental stimuli (Bayne et al. 1976, Koehn & Bayne 1989). To date, stress response by mussel growers has generally been observed at the whole animal level with a reduction in performance such as growth, feeding, condition, and/or quality.

During the growth period of cultured mussels in Atlantic Canada they are completely and continuously submerged in water, and are subject to seasonal and environmental conditions associated with the reproductive cycle, hydrographic events, food avail-

ability, and fluctuating water temperatures. High mortalities have been associated with reproduction, genetics, starvation, and water temperatures, all of which may be influenced by oceanic events (i.e., tidal or wave action, currents) (Freeman & Dickie 1979, Worrall & Widdows 1984, Carver & Mallet 1991, Sephton et al. 1993, Myrand & Gaudreault 1995). As well, during harvesting, mussels can be exposed to a number of environmental factors including abrupt exposure to extreme air or water temperatures, sunlight, rain, and wind, any of which can pose stress on mussels and induce some form of stress response (Warwick 1984, Widdows & Shick 1985, Eertman et al. 1993).

The need to develop earlier and more rapid indicators of stress responses in mussels is ongoing and previous studies have shown changes in physiological characteristics under a number of conditions in feral blue mussels (Bayne 1973, Thompson et al. 1978, Moore et al. 1979, Lowe et al. 1995a, b, Tremblay et al. 1998a, b). Past research on various bivalve species subjected to a number of stressors has shown that the site of earliest detectable stress response is in the lysosome, an organelle of the hemocytes (Moore et al. 1979, Moore 1980, Bayne et al. 1981a, b, Lowe et al. 1995a, b). The NRA can be used to measure alterations in lysosomal stability induced by various stress factors. In their stable form (no stress response), lysosomes will accumulate and retain the neutral red dye for an extended period of time. However, once destabilized (stress response) following a stressor, the lysosomes will coalesce to form larger lysosomal structures and the neutral red dye will leak into the cytosol of the cell through damaged membranes (Moore 1980, Lowe et al. 1995a). The rate of lysosomal changes following the addition of the neutral red dye is indicative of the stress response and can be directly related to the degree of stress being imposed on the mussels.

The objectives of this study are to evaluate the NRA as a stress response indicator in cultivated mussels at the subcellular level (lysosomes), in relation to seasonal and environmental changes and to postharvest handling.

*Corresponding author. E-mail: cyr@mi.mun.ca

MATERIALS AND METHODS

Study Site and Animals

This study was carried out using market size mussels (i.e., >50 mm shell length), which were grown and harvested at Charles Arm, Notre Dame Bay, Newfoundland (49.34 N, 55.28 W) from the period of June 2001 to June 2002. Mussels from this site are >90% *Mytilus edulis* and <10% *Mytilus trossulus* or hybrids (Penney et al. 2001). Charles Arm is a small semiencloded site of an axial length ~3.1 km and connected to the sea by a narrow channel <100 m in width (Penney et al. 2001). Mussels were randomly obtained from mussel socks located on longlines in the middle of the site and shipped in coolers with ice packs to the Marine Institute (St. John's, NL) where they were maintained in flow through raceway systems of seawater at ambient temperature and salinity for at least 2 days prior to experimental use (Harding et al. 2004b).

Experimental Design and Set-up

To assess the seasonal pattern of naturally (physical and biological) induced stress response, mussels were sampled monthly from the culture site from June 2001 to June 2002, with the exception of April 2002 due to ice conditions at the site. Seasonal measurements took into account that there was a natural algae bloom in the late summer to early autumn and in the spring of the year, the normal spawning period in June (assessed by a decline in condition index), and that the water temperature was in the range of 2°C (winter) to 15°C (summer). Measurements of food supply (Chlorophyll-a [Chl-a]), condition index (meat yield), and temperature at the Charles Arm site were monitored on a monthly basis up until November 2001 (see Fig. 2 later) (Nichols et al. 2002). The NRA ($n = 12$ for each monthly sampling), shelf life (3 replicates of 50 mussels for each monthly sampling), and condition index (standard meat yield) were measured against seasonal changes.

To assess the effects of air temperature and extended air exposure on the mussels' subcellular stress response, animals were sampled following harvesting, and following a 2-day recovery in the laboratory (ambient seawater, temperature, and salinity) at 4 times over a year; early summer (June 2001, pre-spawn), late summer (August 2001, post-spawn), early winter (January 2002, recovered), and spring (May 2002, overwinter / early pre-spawn). In one experiment, unprocessed mussels were exposed to air temperatures below, equal to, or above ambient water temperatures, at various times of year. Thermal differentials were in the range of 5°C to 10°C (Table 1). In a second experiment, unprocessed mussels were exposed to air temperatures below, equal to, or above the ambient water temperature of 5°C as observed for that time of the year, with differentials in air temperature of 5°C to 20°C. Mussels were exposed to each temperature for an 8-h period with samples ($n = 12$ mussels) for the NRA being taken at hour 0 as a control, and at hours 4 and 8 for each temperature exposure.

Sampling Procedures

The NRA was performed using the method described by Lowe et al. (1995a) with minor modifications; poly-L-lysine and neutral red stock solutions were increased from 10 µL to 20 µL to enhance cell adhesion to microscope slides and dye intensity in the cells, respectively. Briefly, 0.3 mL of hemolymph was drawn from the posterior adductor muscle into an equal amount of physiological

TABLE 1.

Air temperature exposures (below, equal to, and above ambient water temperature) during the various seasons of early summer (June 2001), summer (August 2001), early winter (January 2002), and spring (May 2002).

Season	Below Ambient Water Temperature (°C)	Ambient Water Temperature (°C)	Above Ambient Water Temperature (°C)
Early summer	2	7	17
Summer	6	16	21
Early winter	-3	2	12
Spring	0	5	15

saline using a 21-gauge needle, with the mixture placed in a siliconized Eppendorf tube and gently inverted. A 40-µL sample of the hemolymph/saline mixture was then placed onto a microscope slide that had been treated with poly-L-lysine. The cells were then left for 15 min to adhere to the slide. A neutral red working solution was prepared from 20 µL of neutral red stock solution and 5 mL of physiological saline, and 40 µL was added to the cell layer and incubated for 15 min in a humidity chamber. After 15 min of incubation, a cover slip was added to the slide and the cells were examined under low light intensity using a compound microscope at ×400 magnification. Cells were examined every 15 min for the first 60 min, then at 90, 120, and 180 min.

For each examination, 25 hemocytes from each mussel sample were examined and given a rating based on 1 of the 4 following characteristics: No stress response = characterized by appearance of tiny pink dots, which are intact lysosomes containing neutral red dye; moderately low stress response = increase in the size of lysosomes due to lysosomal membrane fusion; moderately high stress response = lysosomes appear larger and more faint in color due to fusion of lysosomal membranes and leakage of neutral red dye into the cytosol; high stress response = cytosol is completely tinged pink due to leakage of neutral red dye out of the lysosomes, and only remnants of the lysosomal membranes may be still visible. When higher than 50% of the 25 cells exhibiting high stress response at a particular time point, then the assay was stopped for that mussel and the previous examination time period recorded as the score for NRR.

Shelf life was defined as the time when 5% of mussels in a sample were dead or moribund in a lot. For each monthly sample, 3 replicates of 50 mussels were assessed. Mussels were placed in chilled dry storage of 2°C to 4°C at high humidity, the accepted industry method, each month. Shelf life trials were terminated at day 20, by which time mussels had a strong odor, which would make them unmarketable by industry standards, from initiation of the experiment regardless whether 5% mortality had been reached. Mussel mortality or morbidity (shells fail to remain closed) was assessed daily.

Standard meat yield (CI = condition index) was determined by the following calculation based on 1 kg of live mussels, which were steamed for 10 min (Ibarra et al. 2000):

$$CI = \frac{\text{cooked meat weight}}{(\text{cooked meat weight} + \text{shell weight})} \times 100$$

Data analysis

Data were analyzed using the SPSS (version 10) statistical software. The means (\pm SE), ANOVA, and *post hoc* tests (Tukey's b) were calculated. The level of significance was set $\alpha = 0.05$.

RESULTS

Seasonal Variation

Monthly sampling of mussels showed a seasonal pattern in stress response (Fig. 1A). NRR, a measure of subcellular (lysosome) stress response, showed a significant difference among the months (one-way ANOVA; $F_{(11,130)} = 21.327$, $P < 0.001$), with significant decreases in NRR following spawning events in June 2001, September 2001, and June 2002 (Tukey's b, $P < 0.05$) followed by gradual increases in NRR in the months following spawning.

The average shelf life of mussels examined during each of the monthly samplings showed that there was a significant difference among the months (one-way ANOVA; $F_{(11,24)} = 15.675$, $P < 0.001$) (see Fig. 1B). The average time mussels can survive before they reach 5% mortality was reduced during certain times of the

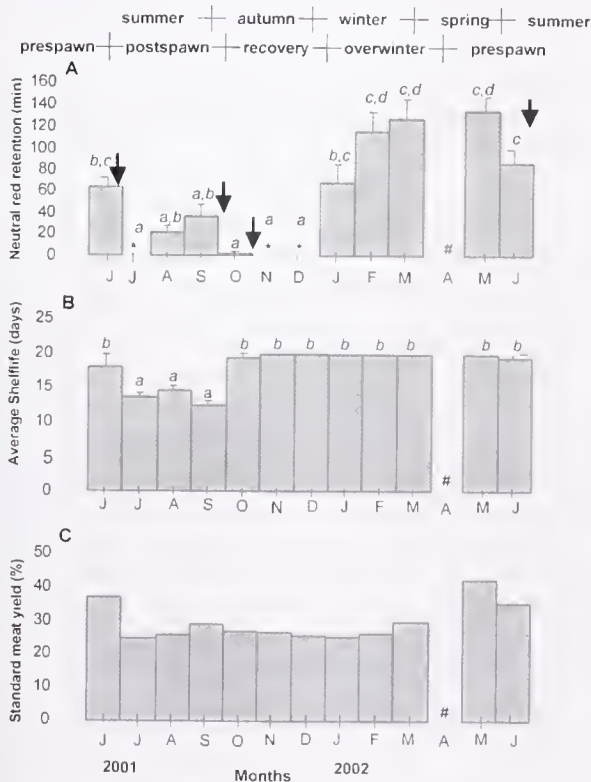


Figure 1. Seasonal variation of neutral red retention, average shelf life, and standard meat yield of *Mytilus* spp. Common letters denote no significant difference among treatments (Tukey's b, $P > 0.05$). (A) Neutral red retention in lysosomes during summer, autumn, winter, and spring conditions. Bars represent the mean \pm SE, $n = 12$ mussels for each month. ANOVA: $P < 0.001$. (* indicates mean = 0); (B) Shelf life in average number of days for 5% mortality to be reached. Each bar represents the mean \pm S.E., $n = 3$ replicates of 50 mussels for each month. ANOVA: $P < 0.001$; (C) Condition index as by standard meat yields. Arrows indicate spawning events. Note: no samples were collected for the month of April (#).

year, especially following the major spawning period in June (see Fig. 1A, B).

The condition of the mussels, as determined by a standard meat yield, showed meat yields decreased following spawning events and gradually increased in months following spawning (see Fig. 1C). Comparison of NRR, shelf life, meat yield and parameters of food availability (Chl-a), temperature, and salinity indicate that NRR and shelf life are influenced by various combinations of these parameters at various times of the year (Fig. 2). NRR was shown to be most affected by the reproductive cycle (determined using standard meat yield) with a significant correlation, $r = 0.87$, and shelf life being influenced inversely by water temperature with a correlation of $r = -0.74$ (Table 2). Regression analysis on the correlation between NRR and meat yield showed significance with $R^2 = 0.75$ ($P = 0.025$), however between temperature and shelf life significance was lower with $R^2 = 0.54$ ($P = 0.9$) (Fig. 3 A,B).

Air Exposures

Experiments examining the rapid, short term exposure of mussels to various air temperatures showed that the NRR response was affected by variation in air temperature, duration of the exposure during harvesting (< 8 h), and seasonal factors (Fig. 4). There was a significant interaction between the seasons and the air temperatures (ANOVA; $F_{(6,371)} = 5.786$, $P < 0.001$), between the seasons and the hours of sampling (ANOVA; $F_{(6,371)} = 6.164$, $P < 0.001$), and between the air temperatures and the hours of sampling (ANOVA; $F_{(4,371)} = 6.084$, $P < 0.001$).

A seasonal pattern of mussel stress response was apparent as NRR (observing time zero for each season) was reduced in months surrounding the spawning period (see Fig. 4). Mussels had low NRR in the postspawning period but this was significantly higher in all other sampling periods. Seasonally, the NRR scores showed that there was no significant difference between mussels in June 2001 (prespawning) and January 2002 (recovered), but that there was a significant difference in mussels in August 2001 (postspawning) and in mussels in May 2002 (overwintered) than all the other seasons respectively (Tukey's b, $P < 0.05$).

In general, results showed that NRR scores of normally submerged mussels subjected to rapid, short term air exposure and held at an air temperature equal to ambient water temperature were significantly higher than any other air exposure temperature. The

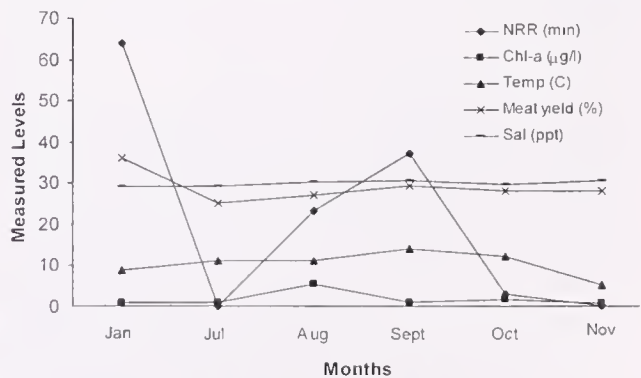


Figure 2. Environmental data and Standard Meat Yield Calculations for mussel aquaculture site (Charles Arm) for months of June 2001 to November 2001. (Data collected with SBE 25 and provided courtesy of Nichols et al. 2002).

TABLE 2.

Correlation values (Pearson r value) for the comparisons among neutral red retention (NRR), standard meat yield (MY), shelf life (SL), chlorophyll-a (chl-a), water temperature (T), and salinity (S) for the period of June 2001 to November 2001 ($n = 6$).

	NRR	MY	SL	Chl-a	T	S
NRR	1	0.867*	-0.167	-0.011	0.178	-0.124
MY		1	0.32	-0.266	-0.167	-0.213
SL			1	-0.178	-0.738*	-0.091
Chl-a				1	0.192	0.162
T					1	-0.102
S						1

* Significant correlation, $P < 0.05$.

air temperatures used in this experiment are given in Table 1. Furthermore, NRR scores of mussels held at air temperatures that were below the ambient water temperature were significantly higher than NRR scores of mussels held at an air temperature that was higher than the ambient water temperature, with the exception of when air below the ambient water temperature was at subzero temperature (Tukey's b , $P < 0.05$). Mussels held at air temperatures that were equal to ambient water temperatures had the highest NRR scores (and less stress) regardless of season or period of time of exposure compared with NRR scores of mussels held at air temperatures either below or above the ambient water temperature (see Figs. 4A, B, and D).

In addition, this experiment showed that short term extended air exposure on mussels reduced NRR in all seasons and temperatures, as NRR scores were significantly different among the hours (ANOVA: $F_{(2,371)} = 136.857$, $P < 0.001$, Tukey's b , $P < 0.05$) (see Fig. 4D). Further examination of air temperature exposure

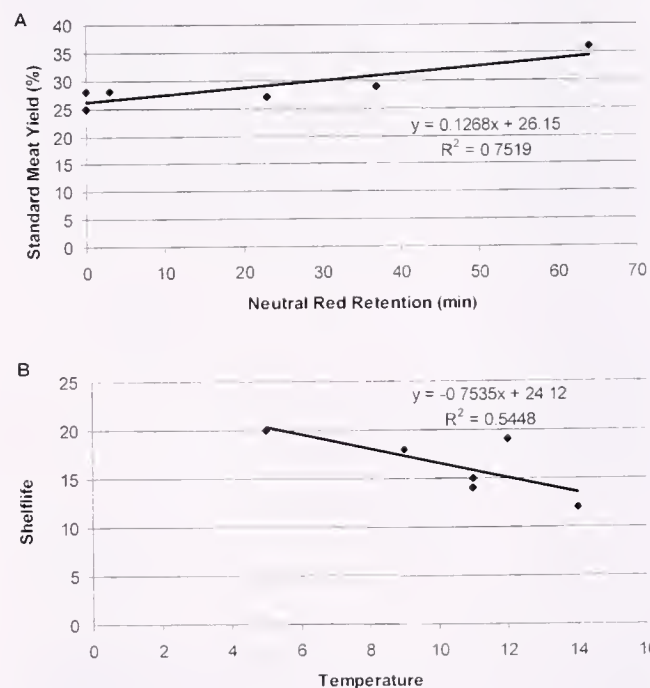


Figure 3. Seasonal parameters found to be significantly correlated for the period of June 2001 to November 2001 inclusive. (A) Neutral red retention and standard meat yield; (B) Temperature and shelf life.

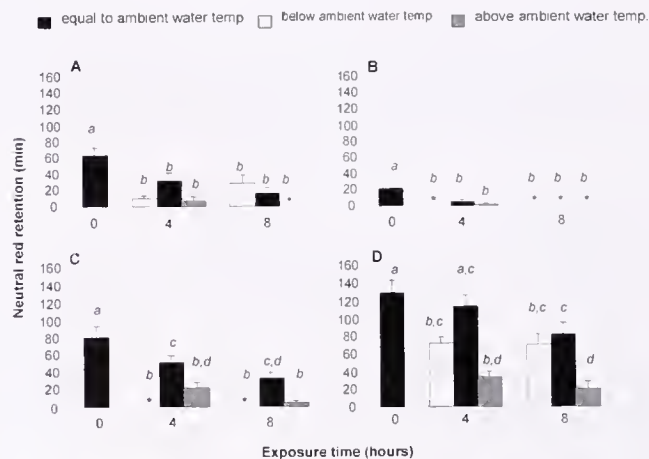


Figure 4. Neutral red retention in lysosomes of *Mytilus* spp. exposed to various air temperatures. Bars represent the mean + SE, $n = 12$ mussels. ANOVA: $P < 0.001$. (* denotes mean = 0). Common letters denote no significant difference among treatments within each season (Tukey's b , $P > 0.05$). (A) Early summer mussels (June 2001, prespawed); (B) Summer mussels (August 2001, postspawed); (C) Early winter mussels (January 2002, recovered); (D) Spring mussels (May 2002, overwintered). See Table 1 for temperature treatments.

showed that NRR declined more rapidly when mussels were exposed to greater differentials in air temperatures in relation to the ambient water temperature of 5°C (Fig. 5).

DISCUSSION

Seasonal and Monthly Variations

The objective of this study is to examine effects of seasonal influences on cultured mussels using and evaluating the NRA as a stress response indicator. The significant difference in NRR among the months and seasons indicates that seasonally related factors were influencing natural and normally induced levels of stress response in mussels. This was an expected occurrence because previous studies have shown that there are differences in a number of physiological factors among the seasons, especially between the warmer and colder months (i.e., summer and winter) (Bayne & Thompson 1970, Moore 1976, Hofmann & Somero 1995, Chapple et al. 1998). Metabolic rate has been shown to differ from summer and autumn to the winter, lysosomal responses have been reported to differ from winter to the summer, and protein levels vary seasonally in feral bivalves (Bayne & Thompson 1970, Moore 1976,

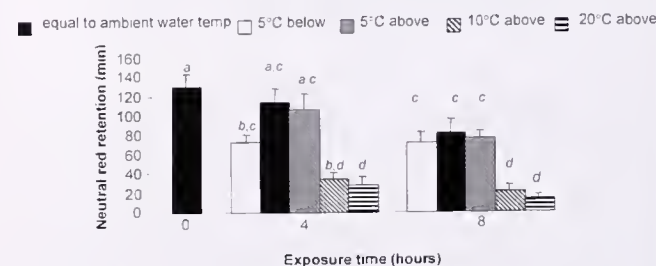


Figure 5. Neutral red retention in lysosomes of *Mytilus* spp. exposed to various air temperatures during spring (May 2002). Bars represent the mean + SE, $n = 12$ mussels. ANOVA: $P < 0.05$. Common letters denote no significant difference among treatments (Tukey's b , $P > 0.05$). Ambient water temperature during this period was 5°C.

Hofmann & Somero 1995, Chapple et al. 1998). Conditions that change with the seasons, including reproductive patterns, water temperatures, and food availability, can lead to major alterations in physiologic mechanisms because the animal strives to maintain homeostasis (Bayne & Thompson 1970, Moore 1976, Hofmann & Somero 1995, Chapple et al. 1998).

The seasonal pattern of natural stress response levels in cultured mussels was shown to be closely related to the reproductive cycle as determined by the standard meat yield (condition index). Spawning events were a factor each time NRR decreased. NRR decreases were observed to significantly correlate with a decrease in condition index. Previous research has shown changes in physiological activities occur during the reproductive cycle in feral mussels including high lysosomal destabilization, higher metabolic demands, lower survival, reallocation of energy, lower oxygen consumption, fluctuations in plasma components, and changes in body glycogen and byssal thread production (Bayne & Thompson 1970, Bayne 1973, Thompson et al. 1978, Worrall & Widdows 1984, Young 1985, Eertman et al. 1993, Myrand & Gaudreault 1995, Tremblay et al. 1998b). The present findings on cultivated mussels support these earlier studies on wild mussel populations.

Naturally induced stress response levels continually decreased throughout the winter months as NRR increased, and only began to decline again in late spring/early summer (June 2002), which was comparable to stress response levels in the previous year (June 2001). Previous studies that examined the reproductive cycle in wild mussels have shown that gametogenesis occurs very slowly during the winter and that its progression is rapid in the spring and early summer, followed by spawning in July (Thompson 1984), thus suggesting a link between stress responses and the breeding cycle.

To further understand seasonal variation of natural stress response levels in mussels, shelf life was also examined as an indicator of stress response at the whole animal level. The shelf life varied little throughout the year with the exception of July 2001 through to September 2001 following the major spawning period. Furthermore, the condition index of mussels was highest prior to spawning and lowest following spawning. These findings support the results of previous studies that showed mussels are in poor condition and have low meat yields following spawning, and they undergo a number of physiological changes during this time that can impede survival ability (Bayne & Thompson 1970, Bayne 1973, Dare & Edwards 1975, Slabyj & Hinkle 1976, Warwick 1984).

Seasonal variations as illustrated by the NRA, shelf life and meat yields can be affected not only by reproduction, but also by seasonal changes in temperature and/or food availability. Seasonal trends in water temperature and food availability (leading to nutritive stress) have been shown to affect byssal production, lysosomal stability, disease (bacterial challenges), and metabolic activity in wild mussels and other bivalve species (Bayne 1973, Young 1985, Hauton et al. 1998, Hauton et al. 2001).

In October and November following spawning, NRR and condition index were significantly lowered, however shelf life continued to increase. Although the NRA measured a high stress response in the months of October and November, rapidly declining water temperatures going into these months, and increased food availability from the autumn phytoplankton bloom prior to these months at the sampling site, Charles Arm, enhanced the survival ability of mussels by reducing the impact of high stress response levels at the subcellular level up to the whole animal level. This study showed that water temperature and shelf life were strongly

linked. Previous studies have shown that mussels are unable to cope with seasonally elevated water temperatures, particularly postspawn, thus a decrease in water temperature would be a benefit to the survivability of the mussels (Sephton et al. 1993, Myrand & Gaudreault 1995). Koehn and Bayne (1989) indicated that with an abundance of energy reserves available, physiological performance of individuals may not be significantly affected by differences in their metabolic efficiency. As suggested by Tremblay et al. (1998), individuals with lower maintenance requirements (related to low water temperature and increased food) would have better survival. In Newfoundland, total particulate matter is highest in summer in more open areas, but is higher in the autumn and spring in small semienclined inlets such as Charles Arm (Thompson 1984, Penney et al. 2001), thus suggesting that food availability was a factor in enhanced, sustained shelf life in the fall, as were seasonally lower water temperatures.

In general, the combination of thermal, nutritive, and reproductive stress influenced the outcome of stress response as assessed by NRA, shelf life and meat yield of mussel. Experiments that evaluated the effects of air temperatures as found during harvesting on barge decks on stress response in mussels corroborated the impacts of these seasonal influences.

By examining stress response using the NRA, in conjunction with the assessment of shelf life and condition index, late autumn (December) through to the spring (May) of the year is the time period when mussels are in the best physiological condition and are least stressed in this subarctic farming site.

Air Exposures

The effects of ambient air temperatures, especially over extended exposure periods, have been shown to be detrimental to mussels (Moore et al. 1979, Widdows & Shick 1985). Understanding the influence of sudden short term air exposure on continuously submerged mussels is important during harvesting on barge decks so as to minimize the impact it may have on the quality of the mussel product. During harvesting mussels are exposed to and maintained in air of various temperatures for relatively short periods of time (<8 h), and they enter an anoxic state bringing about changes in physiological parameters (Moore et al. 1979, Widdows & Shick 1985). These changes are an immediate response to a new environment and in the case of harvesting are a reaction to a short term event that does not allow the mussels time to adapt physiological processes that would later be apparent if time in this condition was extended.

As expected, the results of our experiments showed that exposure to air temperatures equivalent to seasonal ambient water temperatures were least detrimental to mussels, as assessed by the NRA. In general, during summer months, subcellular stress response, as measured by NRR, was high at any exposure temperature and can be attributed to seasonal influences of reproduction. Overall, air temperatures within 5°C of ambient seawater temperatures had little additional effect on mussel stress response, but those mussels exposed to a greater thermal change had a proportionally greater response. Air temperatures that exceeded ambient water temperature by 15°C induced the highest stress response in mussels. Air temperatures vary throughout the year and may harm mussels if they are exposed for extended periods (Widdows & Shick 1985). Moore et al. (1979) demonstrated that in mussels, *Mytilus edulis*, and cockles, *Cerastoderma edule*, an exposure to an increase in air temperature of 10°C induced no change in

lysosomal activity, but did with a 20°C increase, which is in accordance with the present findings. Our study also found that mussels were under high stress during air exposure at subzero temperatures. Past studies have shown that there is a high incidence of mortality in mussels harvested during subzero temperature, and that although *Mytilus edulis* can tolerate freezing up to about -10°C, tissues become injured when 64% of the cellular water is removed to form ice (Williams 1970, Slabyj & Hinkle 1976, Slabyj 1980). In this work, exposure of winter harvested mussels to -3°C air temperature (5°C below ambient water temperature), induced a significant reduction in NRR compared with air temperature equivalent to the ambient water temperature of 2°C.

Experiments revealed that extended exposure to air, no matter what the temperature, increased stress response in mussels during every sampling season. Previous studies support this finding, because aerial exposure has been shown to cause physiological changes, such as altered lysosomal stability in wild *Mytilus edulis* and the clam, *Mya arenaria*, ammonia accumulation in plasma, and increases in the number of hemocytes in mussels (Thompson et al. 1978, Eertman et al. 1993, Tremblay & Pellerin-Massicotte 1997).

Examinations of stress response associated with air exposure suggest that harvesting on barge decks should occur when air temperatures are close to being equivalent to ambient water temperatures, and prolonged period of air exposure should be avoided when air temperatures are below 0°C. If air temperatures exceed a 5°C differential from ambient water temperatures, mussels should be covered to avoid undue stress responses and subsequent reduction in shelf life that may be induced by heating in the sun in summer or freezing in the winter. As well, extended holding of mussels on harvesting decks should be minimized to less than 4 h in duration during any season, unless alternative approaches, such as icing the mussels, can be arranged. The present work suggests that by manipulating or altering standard culture practices we may improve mussel quality and production, and in the end profits for growers.

Shelf Life and Industry Practices

The findings of air exposure on barge decks do not address shelf life issues. Shelf life is a long process extending days as opposed to just hours and provides time for mussels to adapt to anoxic conditions. Exposure to air during harvesting induces initial

physiological changes with the onset of anoxia in mussels and to the air temperatures in which mussels are being harvested, and are not reflective of the survivability of mussels during extended, storage conditions. Concurrent studies with this work examined the effect of various storage conditions, including storage in air, and duration of storage on stress response (i.e., NRA) and shelf life of mussels during the same sampling periods (Harding et al. 2004a).

The NRA can be used successfully to examine stress response levels in lysosomes of cultured mussels, and to determine if various conditions to which cultured mussels are exposed, such as air temperatures, will induce a stress response at the subcellular level in the lysosomes. The NRA can provide information about culture conditions and their effects on mussels, and there are several questions remaining to be answered. For example, can the NRA be used to predict how non market-sized mussels respond to certain conditions while in the water, or during times of grading and socking. As well, the question of genetics and stress response need to be addressed. Different species may have different adaptive abilities and thresholds of tolerance to a variety of conditions. Studies are needed to quantify stress response in mussels of different genetic makeup.

The question of how physiological parameters correlate with the lysosomal stress response changes needs to be examined. The lysosome has been identified as the site of earliest "detectable" change to the state of the mussel, and presuming that the role of lysosomes is largely immunological in hemocytes, it is conceivable that the stress response, as reflected by changes in protein concentrations, O₂ consumption, ammonia levels, and hemocyte activity may be triggered by lysosomal changes. Hence, there is a need to examine the integration of all these processes. Understanding these interactions may lead to development of earlier indicators that the animal has been exposed to stressful conditions and allow for earlier manipulations of conditions to avoid expression of the stress response at the whole animal level.

ACKNOWLEDGMENTS

The authors thank AquaNet, the Canadian Network Centres of Excellence for Aquaculture for partial funding of this project, Memorial University School of Graduate Studies, Marine Institute of Memorial University (especially members of the staff of Centre for Aquaculture and Seafood Development —A. Struthers, J. Nichols, and S. Macneill), Canadian Centre for Fisheries Innovation, National Research Council – Institute for Marine Biosciences and to the staff and management of Thimble Bay Farms.

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SELECTION RESPONSE FOR GROWTH RATE (SHELL HEIGHT AND LIVE WEIGHT) IN THE CHILEAN BLUE MUSSEL (*MYTILUS CHILENSIS* HUPE 1854)

JORGE E. TORO,* ANGÉLICA C. ALCAPÁN, JOHANA A. OJEDA AND ANA M. VERGARA

Instituto de Biología Marina, Universidad Austral de Chile, Casilla 567, Valdivia, Chile.

ABSTRACT The parental stock was taken from the 1999 natural spatfall of *Mytilus chilensis* collected in the Yaldad Bay of southern Chile. From the 22-mo-old cohort 5,688 mussels were monitored for live weight and shell length. Selection was carried out by applying a selection intensity of 1.755 for the trait “live weight”. Five selected lines and five lines of an unselected control group were conditioned in seven 150-L tanks. Juveniles from the 3 selected and 2 control lines were individually tagged and transferred to three geographically distant mussel farms in southern Chile. Live weight and shell length were monitored after 10, 14, 18, and 22 months of age in all experimental mussels. The ANOVA results showed a significant difference, in both traits, between the selected and control groups at every age and location. Realized heritabilities for the trait “live weight” ranged between $h^2 = 0.35$ and $h^2 = 0.54$, whereas those for the trait “shell height” ranged between $h^2 = 0.32$ and $h^2 = 0.49$. Genotype-environment interactions were not apparent for either trait, indicating that similar selection pressures result in similar phenotypic changes for these traits across environments. These results suggest that mass selection for the improvement of the traits live weight and shell height would be effective in the Chilean mussel broodstocks.

KEY WORDS: mussel, *Mytilus chilensis*, selection response, growth, Chile

INTRODUCTION

Mussel aquaculture has enormous potential in southern Chile mainly because the numerous sheltered bays and estuaries along its coastline are free from industrial pollution. Fertilizers and pesticides are rarely applied in local agriculture, and domestic pollution, because of the low human population densities in southern coastal areas. (Winter et al. 1984, Toro & Chaparro 1990).

The Chilean blue mussel *Mytilus chilensis* (Hupe 1854) is an economically important bivalve in southern Chile. Its culture began in 1943 in the area of Quellón located southeast of Chiloé Island (Navarro & Gutierrez 1990, Aiken 1993) and the aquaculture production of this species increased from 3,864 t in 1993 to 41,406 t in 2001 (SERNAPESCA 2002). These values indicate an increase of 971.5 % (37,542 t) for mussel aquaculture between 1993 and 2001. Greater production space (hectares) under cultivation rather than higher outputs per unit area accounts for most of this increased production (Sanchez 2002). However, social, ecological, and economical pressures will eventually promote a greater efficiency of these aquaculture production systems. There are various means to achieve this goal: improved husbandry, nutrition and disease control, and improvement by genetic means. The potential for improving performance by genetic means is unquestionably substantial. There are several reports in the literature addressing the importance and potential of genetics in aquaculture (Moav 1976, Newkirk 1983, Gjerde 1986, Guíñez 1988, Toro & Newkirk 1990, Gjedrem 1999, Koment 2002, Beaumont & Hoare 2003). Increasing production efficiency, reducing generation time, and/or improving survival rate will reduce the negative effects of short growing seasons in high latitudes (Newkirk 1980).

Growth rate has implications for the fitness of individuals and also is considered the most economically important trait to be improved in a selection program (Beaumont & Hoare 2003). Traits of economic importance are usually determined by a large number of genes each having a small contribution and under considerable environmental influence (Newkirk 1980, Lande 1982). Thus, these

traits are best studied through the quantitative genetic theory that reduces the most important features of complex genetic systems to a few variables that can be estimated from phenotypic measurements (Falconer 1989, Lande 1982).

Due to the high market demand for this species, the culture of *M. chilensis* in Chile has been increasing rapidly in the past years (Winter et al. 1984, Navarro & Gutierrez 1990, SERNAPESCA 2002, Sanchez 2002), and because of this mussel aquaculture development, estimates of genetic variation (i.e., heritability) and response to selection pressure are not only important from the ecological point of view but also for practical management purposes (Mallet et al. 1987, Koment 2002). The type of selection scheme that is appropriate depends upon the relative magnitude of the variation of that trait, due to differences in genotype and in environmental factors (Falconer 1989). These mussels are marketable at a shell length of about 55 mm, which is attained after 14 to 16 mo of growth in suspended culture (Winter et al. 1984, Kíno & Valencia 1990). Therefore, a reduction in the time required to attain commercial size (12 mo) could be an important benefit in the culture of this mussel.

Very little has been published on the population genetics of *M. chilensis* and there are no reports on genetic improvement of the Chilean blue mussel. Control of the complete life cycle, which is required for any type of direct genetic improvement (Hershberger et al. 1984), has been accomplished only recently (Toro et al. 2004).

A high intrapopulation variation in the growth rate of *M. chilensis* has been reported in the literature (Winter et al. 1984, Navarro & Gutierrez 1990); however, only a few reports exist on genetic parameters (Toro & Paredes 1996) or genetic manipulation (Toro & Sastre 1995) in *M. chilensis*. A review of the literature shows that heritability estimates in blue mussels have been mainly based on half and/or full-sib correlation (Stromgren & Nielsen 1989, Toro et al. 2004), however, the most appropriate way in a commercial context to determine the heritability of a trait is to carry out selection trials and measure the response to selection for the trait (Beaumont & Hoare 2003). To ensure similar rearing conditions for the selected populations, a control group should also be spawned and their offspring reared in identical environments.

A high heritability value for a particular trait indicates that a

This research was supported by Fondo Nacional de Investigación Científica y Tecnológica, Chile. Project FONDECYT 1010166.

*Corresponding author. E-mail: jtoro@uach.cl

large proportion of the trait is associated with additive gene action, and that genetic progress can therefore be achieved through mass selection (Newkirk et al. 1977). In this study, we consider that h^2 estimates using realized heritability analysis to obtain a more accurate estimation of its values among different rearing locations. To date, there have been no reports on growth and survival of hatchery produced seed of *M. chilensis*. This study represents the first attempt to produce large numbers of juveniles and adults under laboratory conditions and corresponds to a portion of a large-scale breeding program to improve the growth rate of the Chilean mussel *Mytilus chilensis*.

MATERIAL AND METHODS

Adult mussels from a 1999 naturally settled cohort (*Mytilus chilensis* Hupe 1854) were collected from a mussel farm located in Yaldad Bay, Chiloé Island (43°08'S; 73°44'W), southern Chile during October 2001 (prior to the spawning season). At 22 mo of age, a frequency distribution for the trait "live weight" from a random sample ($N = 5,688$) of the cohort of mussels was carried out. The trait presented a normal distribution ($P > 0.05$, Kolmorov Smirnov test, $P > 0.05$). A selection intensity of 1.755 was applied, corresponding to the largest 10% of the trait distribution. A similar number of average-size animals were segregated as control-line parents (Table 1).

Spawning was conducted over a 2-d period. The mussels were kept out of water for about 4–5 h before attempting spawning by thermal shock. They were rinsed in clean seawater and the three selected lines ($N = 200$ each) and 2 control lines ($N = 200$ each) were placed in ten 150-L tanks with filtered (1- μ m) and UV-treated sea water (FSW) at 18°C. They were then continuously monitored until they spawned at which time they were removed. The day after the spawning the water of each tank was filtered through a 45- μ m mesh. The embryos collected from each family were placed into a 200-L fiberglass tank containing 1- μ m filtered and UV-treated fresh seawater (FSW) at $16 \pm 1^\circ\text{C}$, and a density of 100 individuals per mL.

After 48 h the density was adjusted to 5 larvae per mL. A high cell concentration (100,000 cells/mL) of the microalgae *Isochrysis galbana* (Parke 1949) and *Chaetoceros gracilis* (Schuett) were used as food (Toro & Paredes 1996). Every day the water in each tank was passed through a 45- μ m "nitex" screen to retain the larvae. Each beaker was rinsed with fresh water followed by seawater. The larvae were then resuspended in FSW and algal food was added daily at the desired cell concentration.

TABLE 1.

Mytilus chilensis. Basic statistics for the traits "live weight" (g) and "shell height" (mm) for the 5,688 individuals from the 22 months old cohort used as a base population in the selection program.

N	Population 5688	Control 400	Selected 600
Live weight			
Mean (g)	18.47	18.42	26.50
Standard Deviation	4.27	0.89	2.54
Coefficient of Variation (%)	23.11	4.83	9.50
Shell height			
Mean (mm)	63.05	63.41	69.73
Standard Deviation	5.24	3.11	3.95
Coefficient of Variation (%)	8.31	4.90	5.66

The juveniles from each selected and control lines were settled on netlon mesh and after 2 weeks of growth in the laboratory, transferred to the field (Quetalmahue) until they were ready for tagging (5 mm). After 15 weeks of growth in the field, the juveniles from each line were individually labeled and transferred randomly in pearl nets to 3 different aquaculture farming sites. Hueihue Bay, located 500 m from a commercial oyster farm; and Quetalmahue Gulf and Putemún Channel, located around a mussel farm, all of them within the Chiloé Island (Fig. 1).

Live weight and shell height were monitored at 10, 14, 18, and 22 mo of age using an A&D portable balance (± 0.01 g) and vernier calipers (± 0.1 mm).

Analysis of Variance (ANOVA) including the effects of location, group (selected and control), and their interactions were carried out for live weight and shell height at the four life stages using the SYSTAT 5.1 statistical package (Wilkinson 1991). Log-transformations were used to normalize the data where appropriate. The response to selection and realized heritability estimates and their standard errors were calculated according to Falconer (1989), Becker (1992), and Hadley et al. (1991). A statistical comparison ($P \leq 0.05$) of differences in heritability values (Student's t test) was done.

RESULTS

Growth data at 10, 14, 18, and 22 mo of age for the traits live weight and shell height at 3 locations are presented in Table 2. From the mean of each group (control and selected) it can be seen that there are differences in both traits among the three different locations. The ANOVA results (Table 3) show that for live weight

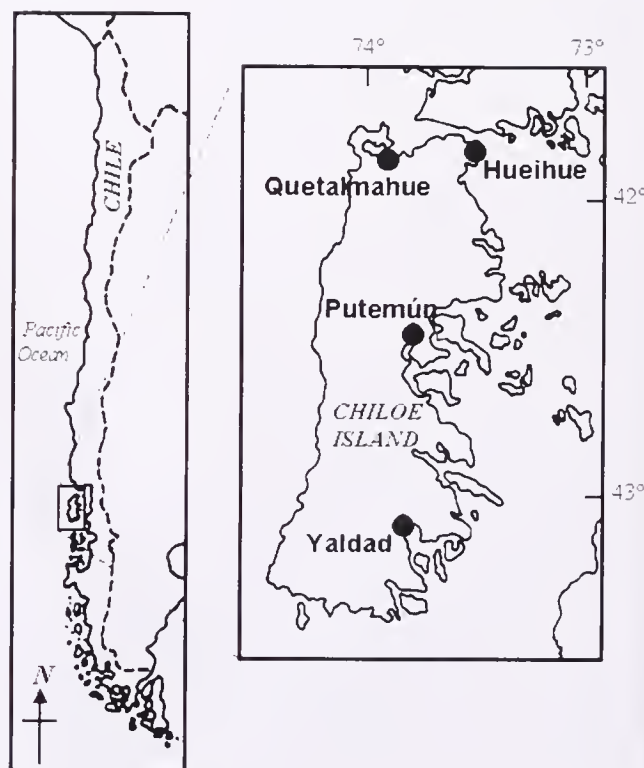


Figure 1. *Mytilus chilensis*. Aquaculture farms (Hueihue, Quetalmahue, Putemún) where the experimental mussel grew up and location of the natural population used as a source of the parental stock (Yaldad) (●).

TABLE 2.

Mytilus chilensis. Basic statistics for the traits "shell height" and "live weight" in the F1 of the selected and control groups at 10, 14, 18, and 22 months old maintained in the 3 mussel farms: Hueihue, Putemún, and Quetalmahue, (mean: average; SD: standard deviation).

Farm	Group	Age (months)	Live Weight (g)		Shell Height (mm)	
			Mean	SD	Mean	SD
Hueihue	Control	10	5.35	2.22	36.73	5.39
		14	9.00	3.25	44.68	2.28
		18	11.81	3.10	49.28	4.55
		22	19.20	5.29	56.37	4.35
	Selected	10	6.55	1.51	40.89	4.50
		14	10.36	2.36	48.23	4.30
		18	12.30	2.69	52.78	4.15
		22	22.34	4.18	58.42	4.28
Putemún	Control	10	5.53	2.35	37.60	5.87
		14	9.02	2.89	45.12	5.13
		18	12.10	3.01	49.90	4.37
		22	18.77	6.16	56.50	5.47
	Selected	10	6.07	1.88	39.76	5.10
		14	9.15	2.40	46.03	5.18
		18	12.41	3.17	50.72	4.81
		22	22.80	4.60	59.93	5.75
Quetalmahue	Control	10	7.58	2.52	42.68	5.39
		14	12.38	3.39	50.27	5.31
		18	15.20	3.62	54.20	4.76
		22	21.52	4.48	58.24	5.04
	Selected	10	8.14	2.11	44.99	4.50
		14	13.50	2.81	52.50	4.54
		18	16.20	3.37	55.90	4.55
		22	24.38	4.09	61.28	4.37

TABLE 3.

Mytilus chilensis. Analysis of variance (ANOVA) for the traits "shell height" and "live weight" at 10, 14, 18, and 22 months old.

Effect	df	Shell Height		Live Weight	
		MS	P	MS	P
10 Months					
Location	2	4586.79	<0.05	673.52	<0.05
Group	1	368.00	<0.05	22.10	<0.05
Location * Group	2	75.93	>0.05	8.47	>0.05
Error	1507	29.33		5.32	
14 Months					
Location	2	2727.43	<0.05	1047.90	<0.05
Group	1	212.36	<0.05	54.22	<0.05
Location * Group	2	66.05	>0.05	14.71	>0.05
Error	1016	26.66		9.70	
18 Months					
Location	2	1552.06	<0.05	11.34	<0.05
Group	1	96.84	<0.05	40.85	<0.05
Location * Group	2	46.96	>0.05	7.44	>0.05
Error	719	20.81		10.52	
22 Months					
Location	2	348.51	<0.05	382.84	<0.05
Group	1	1465.70	<0.05	2030.38	<0.05
Location * Group	2	30.38	>0.05	22.92	>0.05
Error	747	23.75		23.26	

df, degrees of freedom; MS, mean squares.

TABLE 4.

Mytilus chilensis. Realized heritability estimates (h^2) and their standard errors (\pm SE) for the traits "shell height" and "live weight" at 22 months old grown at 3 geographically separated mussel farms.

	Shell Height		Live Weight	
	h^2	SE	h^2	SE
Hueihue	0.32	± 0.017	0.38	± 0.020
Putemun	0.49	± 0.024	0.54	± 0.018
Quetalmahue	0.48	± 0.016	0.35	± 0.015

and shell height, there is a significant effect of "location" (three locations) and "group" (selected and control) sources of variance. There were no significant effects of the location by group interaction term. A significant response to selection for the direct selected trait "live weight" was found at all ages monitored (see Table 3). The standardized responses fluctuated between 0.77 and 0.05 units of SD from the control. A correlated response for the trait 'shell height' was detected (see Table 3). The response measured as standardized Index did not differ significantly among the different localities ($P > 0.05$).

The realized heritability estimates for the traits "live weight" and "shell height" at 22 months of age exhibited values significantly greater than zero in all three locations (Table 4). Heritabilities for live weight did not differ significantly between Hueihue and Quetalmahue locations ($P > 0.05$) while Putemún and Quetalmahue showed similar heritabilities for shell height ($P < 0.05$); therefore even when growth rates differed significantly among environments, the additive genetic variance did not vary from location to location, with the exception of Putemún and Hueihue for live weight and shell height respectively.

DISCUSSION

Live weight and shell height varied significantly among environments in which the offspring were raised (see Table 2 and 3). This implies that environmental variation plays a large role in determining these growth traits. The location \times group interaction (G \times E) was not significant for either of the two traits (see Table 3), meaning that although there was significant variation among environments for each trait, the overall rank of each group in each environment was unchanged across sites.

There was a significant and steady response to selection in both traits across the environments. These results are in accordance with those reported by Crenshaw et al. (1991), for *Argopecten irradians concentricus*; Bustos et al. (1991) and Toro et al. (1994), for *Ostrea chilensis*; and Nell et al. (2000), for *Saccostrea glomerata* were a positive response to selection was found. Toro and Newkirk (1991) working with *O. chilensis* reported also a significant response to

reduce the same trait, comparing a low selected line against an unselected control line.

The realized heritability estimates in both traits 22 months of age (see Table 4), were significant across the three environments measured, although, no significant genotype \times environment interaction for these traits were detected (see Table 3). Therefore, a selection intensity applied to these traits results in predictable changes in the phenotype values of the traits among environments (Falconer 1989). There are no reports on realized heritabilities on adult mussels, however, the heritability values obtained here are lower than those reported by Newkirk & Haley (1982), in *Ostrea edulis*; Lannan (1972), in *Crassostrea gigas*; Stromgren & Nielsen (1989) and Losee (1978), in *Mytilus edulis*. However, they are in accordance with those reported by Toro & Newkirk (1990), in *O. edulis*; Toro & Newkirk (1991), in *O. chilensis*; Wada (1986), in *Pinctada fucata martensii* ($h^2 = 0.47$ & 0.35 for shell width and shell convexity respectively); Hadley et al. (1991), in *Mercenaria mercenaria* ($h^2 = 0.43$ for growth rate); and also in line with values for production traits in livestock (Van Vleck 1987).

Using the realized heritability values obtained and applying a selection intensity of 1.755 that is equivalent to select the higher 10% of the population for the trait, the estimated mean change in live weight ranges between 2.6 % to 4.0 % and for shell height between 2.9 % to 4.5 % per generation. These values of response to selection for increasing the traits are lower than those given by Mallet et al. (1986) and Stromgren & Nielsen (1989) for *M. edulis* and Newkirk & Haley (1982) for *O. edulis*; these authors reported a large response to selection for growth to market size with an average of 23 % of gain over the controls.

The correlated response found for the trait "shell height" suggests a positive genetic correlation between the two traits. Very high genetic correlations between live weight and shell height have been reported ($r_a = 0.995$) for *O. edulis* (Toro & Newkirk 1990) and for *M. edulis* juveniles (Nielsen 1985).

The estimates of heritability for traits of economic importance such as live weight and shell height is essential in a long term breeding program to enhance the growth rate of the Chilean mussels. The quantitative genetic parameter estimates should be obtained under natural conditions and their constancy measured among different environments to be able to predict potential responses to selection (Mitchell-Olds & Rutledge 1986). Heritability values are essential for the purpose of predicting the genetic gain by selection. In common with previous studies of bivalves, our experiments demonstrate significant genetic variation in live weight and shell height, which may have implications for the biology of natural populations of *M. chilensis* as well as for commercial culture.

ACKNOWLEDGMENTS

We thank Instituto Fomento Pesquero, Centro Hueihue, for all the laboratory facilities to carry out this project.

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EVIDENCE FOR THE PRESENCE OF THE PORTUGUESE OYSTER, *CRASSOSTREA ANGULATA*, IN NORTHERN CHINA

*SYLVIE LAPÈGUE,¹ FREDERICO M. BATISTA,² SERGE HEURTEBISE,¹ ZINIU YU³ AND PIERRE BOUDRY¹

¹Laboratoire de Génétique et Pathologie, IFREMER, 17390 La Tremblade, France; ²IPIMAR/CRIPSul, Av. 5 de Outubro, 8700-305, Olhão, Portugal; ³College of fisheries, Ocean University of China, Qingdao 266003, People's Republic of China

ABSTRACT The Pacific oyster, *Crassostrea gigas* (Thunberg), and the Portuguese oyster, *C. angulata* (Lamarck), are two closely related taxa. Although these two taxa were both introduced from Asia into Europe, one (*C. gigas*) was voluntarily introduced in the early 1970s, whereas the other (*C. angulata*) was presumed to be present in Europe for at least four centuries, but nearly disappeared because of disease. Few *C. angulata* populations remained in southern Portugal, Spain and Morocco and their putative origin was traced in Taiwan. The present paper reports evidence for its presence in Northern China. We reanalyzed recently published mitochondrial cytochrome oxidase C subunit I (COI) sequence data from presumed Dalianwan oysters (*C. talienwhanensis*) and compared them with those of *C. gigas* and *C. angulata*. Additionally, two new *C. angulata* haplotypes from Portugal were identified. The results clearly showed that some of the *C. talienwhanensis* sequences cluster with *C. angulata* sequences. The relative divergence between *C. gigas*, *C. angulata*, and *C. talienwhanensis* haplotypes indicated that *C. angulata*-like oysters are present in northern China. This opens new perspectives in terms of genetic resources and population genetics of *C. gigas* and *C. angulata*, two oyster species of aquacultural importance.

KEY WORDS: cupped oysters, *Crassostrea angulata*, *Crassostrea gigas*, *Crassostrea talienwhanensis*, cytochrome oxidase C subunit I gene, phylogeography

INTRODUCTION

The phenotypic plasticity of oysters and their wide range of distribution are known to have led to numerous taxonomic misclassification or redundancy in species names. However, during the last decade, molecular tools have contributed in highlighting and resolving several of these cases. For example, Anderson and Adlard (1994) proposed that *Saccostrea commercialis* and *S. glomerata* should be regarded as synonymous taxa based on rDNA internal transcribed spacer sequence data. More recently, Kenchington et al. (2002) suggested that *Ostrea edulis* and *O. angasi* are conspecific. In addition, O'Foighil et al. (1999) confirmed the transoceanic range (New Zealand and Chile) of *O. chilensis* using mitochondrial COI sequence data and proposed that dispersal by rafting was the most likely explanation for this distribution. Similarly, the mangrove oyster *Crassostrea gasar* was shown to be present not only along the coasts of Western Africa but also along the Atlantic coasts of South America where some specimens had been wrongly described as *C. rhizophorae* (Lapègue et al. 2002).

In this context, the relative taxonomic status of the Portuguese oyster, *C. angulata*, and the Pacific oyster, *C. gigas*, may be considered as a case study. *C. gigas* and *C. angulata* were classified as two different species by Thunberg in 1793 and Lamarck in 1819, respectively. This classification was chiefly due to apparently separated geographical distribution of the two species, because *C. angulata* was described in Europe and *C. gigas* in Asia. However, following morphologic comparison (Ranson 1948), experimental hybridization (reviewed by Gaffney & Allen 1993, Huvet et al. 2001, Huvet et al. 2002) and allozyme data (Mathers et al. 1974, Buroker et al. 1979, Mattiucci & Villani 1983), the authors concluded that there was only a single species, grouping Portuguese and Pacific oysters. Yet, significant phenotypic differences between the two taxa were observed, in terms of production yield (Bougrier et al. 1986, Héral 1986, Parache 1989, Soletchnik

et al. 2002), and eco-physiologic characteristics (His 1972, Goulletquer et al. 1999, Haure et al. 2003). Furthermore, differences have now been observed (1) from karyotype analyses (Leitão et al. 1999b) although these two taxa exhibited a close genetic similarity in comparison with other cupped oyster species (Leitão et al. 1999a); (2) in the genetics of the two taxa based on the mitochondrial COI gene (Boudry et al. 1998, O'Foighil et al. 1998) and nuclear microsatellites (Huvet et al. 2000).

The introduction of the Pacific oyster was relatively well documented because it was a recent voluntary introduction. Hence, the introduction of *C. gigas* from Japan into Europe was made in the early 1970s to replace the Portuguese oyster in the shellfish industry (Grizel & Héral 1991) that nearly disappeared probably due to an iridoviral disease (Comps 1969). As indicated by its common name, the Portuguese oyster, was believed to originate from Portugal or at least southern Europe. However, results from nuclear and mitochondrial DNA studies (Boudry et al. 1998, O'Foighil et al. 1998, Huvet et al. 2000) suggested an explanation for the separated geographical distribution of these genetically closely-related taxa by supporting the hypothesis of the introduction of *C. angulata* from Asia (and more precisely Taiwan) to the Portuguese coast by merchant ships during the 16th century. Until now, no *C. angulata* specimens were observed in any other Asian location (see Lam et al. 2003, Boudry et al. 2003).

China is the country with the largest *C. gigas* production (in 1997: Mainland China: 2.3 10⁶ metric tonnes, Taiwan: 24 10³ metric tonnes, Hong Kong: 66 metric tonnes, according to FAO, 1999). However, Guo et al. reported in 1999 that it is not *C. gigas*, but another species, *C. plicatula*, that accounts for the main 50% to 60% of the production, *C. ariakensis* for 20% to 30% and *C. gigas* for only 10% to 20%. Qi (1989) also cited the Dalianwan oyster (*C. talienwhanensis*) as of commercial importance after the Zhe oyster (*C. plicatula*) and the Suminoe oyster (*C. ariakensis*) (Qi 1989). Many other different taxa have been reported along Chinese coasts and species identification is often uncertain. In Northern China, *C. talienwhanensis*, *C. plicatula* and *C. gigas*

*Corresponding author. E-mail: Sylvie.Lapegue@ifremer.fr

were considered as sibling species (Liu & Dai 1998). In the East and South China, at least nine species have been described (Bernard et al. 1993). Recently, a new species was described in Hong Kong (Lam & Morton 2003, Boudry et al. 2003). Finally, a recent study based on mitochondrial DNA sequences showed that *C. talienwhanensis* specimens from Northern China were genetically close to *C. gigas* specimens which suggested that the Dalianwan and Pacific oysters belong to the same species (Yu et al. 2003). Similarly, their results suggested that *C. plicatula* and *C. ariakensis* are closely related.

In the present paper, we compared DNA sequence data of *C. gigas*, *C. talienwhanensis*, and *C. angulata* to establish their genetic relationships. To do so, we reanalyzed the mitochondrial COI sequence data of *C. talienwhanensis* specimens from Yu et al. (2003) and compared with those of known haplotypes from *C. gigas*, *C. angulata* (Boudry et al. 1998) and two newly identified *C. angulata* haplotypes.

MATERIALS AND METHODS

New *Crassostrea angulata* Sequence Data

A total of 218 cupped oysters from Portugal were sampled in October of 2002 from 2 locations, 109 in the Sado estuary (Monte da Pedra site: 38°25'N, 8°39'W) and 109 in the Mira estuary (Roncão Velho site: 37°42'N, 8°44'W). DNA extraction of ethanol-preserved gill fragments was performed by a phenol/chloroform method, as described by Moore (1993). A partial COI fragment was amplified using the primers and conditions detailed in Folmer et al. (1994). Polymorphism was first studied using restriction enzymes as described by Boudry et al. (1998). Some of these PCR products were sequenced as described by Boudry et al. (2003). All the novel sequences have been submitted to the EMBL nucleotide sequence database.

DNA Sequence Analysis

The COI sequences of the new haplotypes, together with some sequences already obtained for *Crassostrea gigas* and *C. angulata*

(Accessions AJ553901, AJ553902, AJ553903, AJ553904, AJ553905; Boudry et al. 2003), *C. virginica*, *C. ariakensis*, and *C. sikamea* (Accessions AF152566, AF152569, AF152568; O'Foighil et al. 1998) and *C. talienwhanensis* (Yu et al. 2003) were aligned with CLUSTALW (Thompson et al. 1994). Pairwise sequence divergences between species were estimated with the DNADIST program in PHYLIP (Felsenstein 1989) according to Kimura's two-parameter model (Kimura 1980). Phylogenetic analyses were conducted using the program FITCH. Bootstrap analysis with 100 replicates was performed with the SEQBOOT and CONSENSE programs.

RESULTS

PCR-amplified fragments from the COI gene were obtained for 218 individuals from the newly sampled Portuguese populations. The PCR-RFLP analysis detected 2 new haplotypes, which were called F and G here. Haplotype G lacked a *Sau3A* restriction site when compared with the others haplotypes of *Crassostrea gigas* and *C. angulata* described by Boudry et al. (1998). For haplotype F a new restriction site was observed using *MseI* when compared with the same haplotypes. These new haplotypes are rare, each one being present in one of the two populations: the frequency of haplotype F is 5% in Mira and the frequency of haplotype G is 2% in Sado. The COI sequences of haplotypes F and G, together with haplotype J—that had been described by Boudry et al. (2003), but not sequenced—were respectively registered as Accessions AY397685, AY397686, and AY455664. We compared the sequences of these haplotypes, F, G, and J, with those of haplotypes from *C. gigas* (haplotype C, D, and E; Boudry et al. 2003), *C. angulata* (haplotype A and B; Boudry et al. 2003), and *C. talienwhanensis* (haplotype talienw1, talienw2, talienw3, talienw4, and talienw5; Yu et al. 2003). The multiple alignment showed that haplotypes C and talien1 had exactly the same sequence. The distances computed after this alignment is presented in Table 1. The phylogenetic tree obtained from sequence divergence of the COI fragment according to Kimura's model (Kimura

TABLE 1.
Pairwise sequence divergences, for the mt COI DNA fragments (*C. angulata* haplotypes are in bold).

	Haplotype E	Haplotype C	Haplotype D	talienw2	talienw3	Haplotype A	Haplotype B	Haplotype G	Haplotype J	Haplotype F	talienw4	talienw5	<i>C. sikamea</i>	<i>C. ariakensis</i>	<i>C. virginica</i>
Haplotype E	0														
Haplotype C	0.0036	0													
Haplotype D	0.0055	0.0018	0												
talienw2	0.0055	0.0018	0.0036	0											
talienw3	0.0055	0.0018	0.0036	0.0036	0										
Haplotype A	0.0279	0.0241	0.0222	0.0260	0.0260	0									
Haplotype B	0.0337	0.0299	0.0279	0.0318	0.0318	0.0055	0								
Haplotype G	0.0317	0.0279	0.0260	0.0299	0.0299	0.0036	0.0055	0							
Haplotype J	0.0375	0.0337	0.0317	0.0356	0.0356	0.0091	0.0110	0.0091	0						
Haplotype F	0.0317	0.0279	0.0260	0.0298	0.0298	0.0110	0.0129	0.0110	0.0166	0					
talienw4	0.0298	0.0260	0.0279	0.0279	0.0279	0.0055	0.0073	0.0055	0.0073	0.0129	0				
talienw5	0.0279	0.0241	0.0260	0.0260	0.0260	0.0036	0.0055	0.0036	0.0091	0.0110	0.0018	0			
<i>C. sikamea</i>	0.1051	0.1053	0.1031	0.1075	0.1075	0.1007	0.0986	0.1007	0.0984	0.1050	0.1029	0.1007	0		
<i>C. ariakensis</i>	0.1623	0.1625	0.1650	0.1650	0.1601	0.1652	0.1725	0.1701	0.1723	0.1723	0.1676	0.1652	0.1679	0	
<i>C. virginica</i>	0.2590	0.2538	0.2512	0.2565	0.2512	0.2513	0.2567	0.2567	0.2569	0.2592	0.2594	0.2567	0.2627	0.2789	0

talien1 and haplotype C have the same COI sequence.

1980) is given in Figure 1. The 12 haplotypes clustered into 2 groups (100% bootstrap *P* value) with a divergence varying from 2.2% to 3.7% depending the pairs of haplotypes being compared. One group encompassed the *C. gigas* haplotypes (C, D, and E) and the *C. talienwhanensis* haplotypes (talienw1, talienw2, and talienw3). The second group included the *C. angulata* haplotypes (A, B, J, F, and G) and the *C. talienwhanensis* haplotypes (talienw4 and talienw5). The nucleotide divergence within the first group varies from 0.2% to 0.5% and in the second group from 0.2% to 1.7%. *C. sikamea* and *C. ariakensis*, two other Asian cupped oysters species, respectively exhibited about 10% and 16% divergence with the *C. gigas* and *C. angulata* haplotypes. The American oyster, *C. virginica*, showed about 26% divergence with the *C. gigas* and *C. angulata* haplotypes and was considered as an outgroup.

DISCUSSION

The geographic distribution of the closely related taxa *Crassostrea gigas* and *C. angulata* in southwestern Europe is now well documented, so is the genetic variability within the populations and the genetic differentiation between the populations. *C. gigas* was observed in northern Europe bordered by the headland of northern Spain (La Corogne) in the south. *C. angulata* was observed in southern Spain, Portugal, and Morocco (Boudry et al. 1998, Fabioux et al. 2002, Huvet et al. 2004). According to the

grouping of the haplotypes observed here, haplotype F and G, detected in the new samples from the Mira and Sado Portuguese populations, can be considered as two new *C. angulata* haplotypes. This is confirmed by the divergence values that are of the order of those reported by Boudry et al. (2003): less than 0.5% for the *C. gigas* group compared with 0.2% to 0.5% in this study, and less than 1.1% for the *C. angulata* group compared with 0.2% to 1.7% in this study. When observing the results for the *C. talienwhanensis* haplotypes from Yu et al. (2003), talienw1 (identical to haplotype C), talienw2 and talienw3 are grouped with the *C. gigas* haplotypes, and talienw4 and talienw5 with the *C. angulata* haplotypes. Consequently, oysters of talienw1, talienw2 and talienw3 can be considered as *C. gigas*, as proposed by Yu et al. (2003), and the others (oysters of talienw4 and talienw5) can be considered as *C. angulata*. This partly confirms that the Dalianwan oyster, described by Zhang and Lou (1956), is another name for the Pacific oyster in China (Li & Qi 1994), but also supports that *C. talienwhanensis* being considered as *C. angulata*. After the evidence of the presence of *C. angulata* in Taiwan (Boudry et al. 1998), this species is now found existing in the northern China (Dalian, Liaoning province and Rongcheng, Shandong province) suggesting a broader Asian geographical distribution. Additionally, it should be noted that the cupped oysters specimens found in Hong-Kong (Lam et al. 2003, Boudry et al. 2003) and considered as belonging to a putative new taxa, cannot be considered as *C. talienwhanensis*.

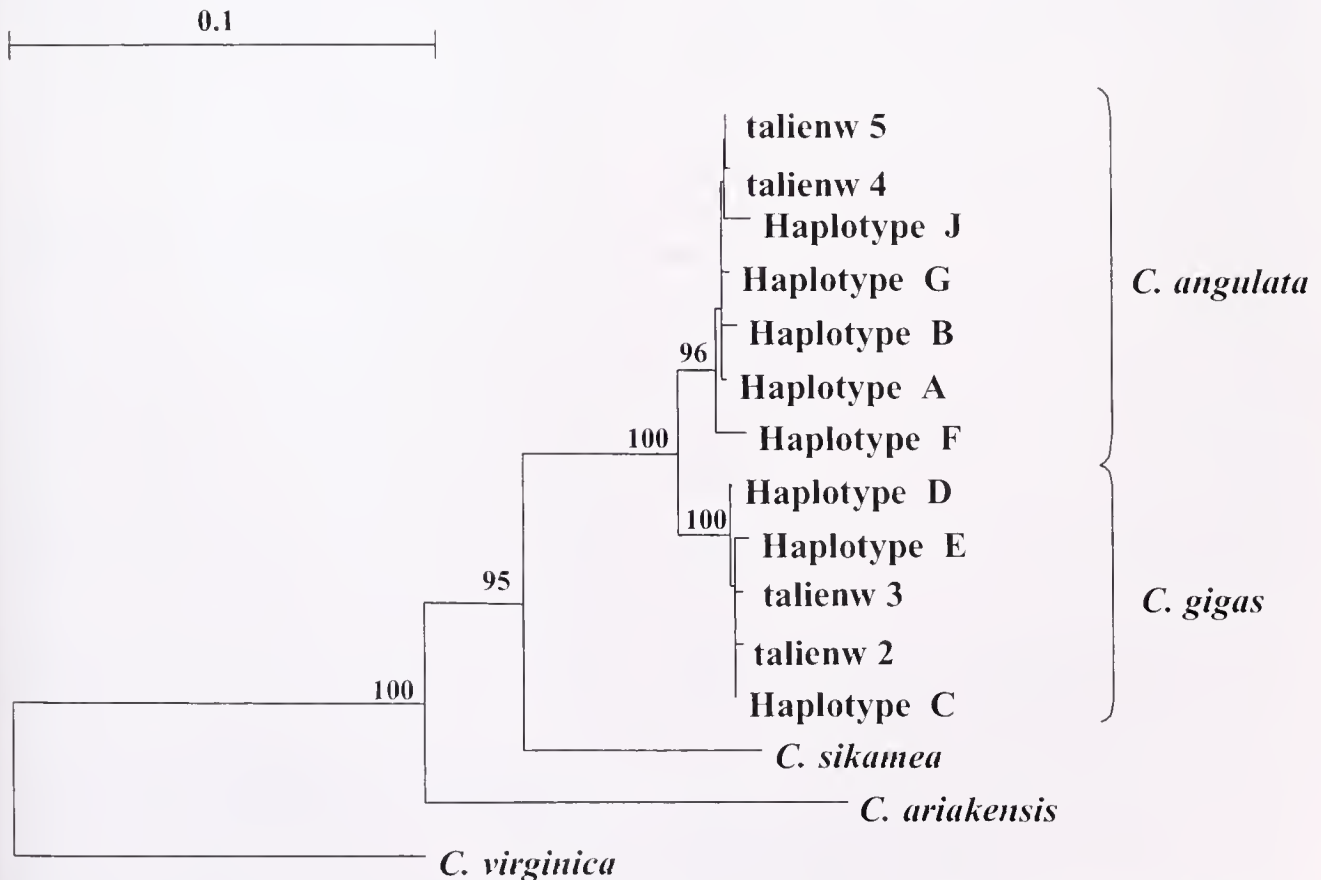


Figure 1. Phylogenetic trees obtained from sequence divergence of a 551 nucleotide mitochondrial COI DNA fragment according to Kimura's model (Kimura 1980) for *C. gigas*, *C. angulata*, and *C. talienwhanensis* haplotypes. talienw1 to 5 correspond to the haplotypes described by Yu et al. (2003). The sequences of haplotypes A, B, C, D, and were described in Boudry et al. (2003). The sequences of *C. ariakensis*, *C. sikamea* and *C. virginica* were obtained from O'Foighil et al. (1998). The sequences of haplotypes F, G, and J were described in this study. *C. virginica* was used as an outgroup. Numbers on the branches indicate bootstrap *P* values.

In total, 11 *C. angulata* COI haplotypes (A, B, and J from Boudry et al. 1998; angul1, angul2, angul3, and angul4 from O'Foighil et al. 1998; talienw4 and talienw5 reanalysed from Yu et al. 2003; F and G in this study) and 5 *C. gigas* COI haplotypes (C, D, and E from Boudry et al. 1998; talienw1 (identical to haplotype C), talienw2, and talienw3 reanalysed from Yu et al. 2003) have now been described. Interestingly, using relatively equivalent amount of research on both taxa, the level of variability of *C. gigas* appears to be lower than that of *C. angulata* for the COI sequence. Furthermore, studies based on allozyme data by Buroker et al. (1979) also showed a high genetic variability in *C. angulata*, from Portuguese populations, relative to other *Crassostrea* species. More data are needed from other mitochondrial and nuclear markers, but this high genetic variability observed in *C. angulata* opens interesting perspectives for the development of conservation programs for this taxa in Europe. Consequently this also underlines the importance of *C. angulata* as a potentially useful genetic resource for *C. gigas* aquaculture.

Although the two 16S haplotypes described for the 10 *C. talienwhanensis* individuals from Yu et al. (2003) study were each found in 2 different sampling locations (Dalian & Rongcheng), the 5 COI haplotypes (talienw1 to talienw5) distribution indicated that

C. gigas and *C. angulata* co-occur in the two sampled locations. Hence, one individual has the talienw1, one the talienw2, one the talienw3, four the talienw4, and three the talienw5 haplotypes. This result needs to be confirmed, because the number of samples (5 in each location) is low. It would be of particular interest to focus on the sympatric status of these two taxa in this region as it was done in Southern Europe. In Portugal, there was evidence for hybridization between *C. angulata* and *C. gigas* in the wild where the two taxa are in contact due to recent transportation of *C. gigas* stocks for aquacultural production (Huvet et al. 2004). An extensive sampling protocol is clearly needed in Northern China to investigate this hybridization in the native region of these two taxa, and, more generally, their relative evolutionary history.

ACKNOWLEDGMENT

The authors thank H. McCombie for help with the English. This work was made possible by an FCT grant (SFRH/BD/8972/2002) and a Marie Curie Training fellowship (PLUDAMOR, QLK5-CT-2000-60036) to F. M. Batista. This work was partly supported by the Région Poitou-Charentes (Convention N°2001-RPC-A-212).

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THE DISTRIBUTION AND ECOLOGICAL EFFECTS OF THE INTRODUCED PACIFIC OYSTER *CRASSOSTREA GIGAS* (THUNBERG, 1793) IN NORTHERN PATAGONIA

MAURICIO ESCAPA,^{1,2,3*} JUAN PABLO ISACCH,^{1,2} PEDRO DALEO,^{1,2} JUAN ALBERTI,^{1,2} OSCAR IRIBARNE,^{1,2} MONICA BORGES,^{3,4} EDER P. DOS SANTOS,³ DOMINGO A. GAGLIARDINI² AND MARIO LASTA⁵

¹Departamento de Biología (FCEyN), Universidad Nacional de Mar del Plata, CC 573 Correo Central, B7600WAG, Mar del Plata, Argentina; ²Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); ³Instituto Argentino de Oceanografía (IADO, CONICET), Florida 8000 Complejo CRIBABB Edificio E1, 8000, Bahía Blanca, Argentina; ⁴Convenio Subsecretaría de Actividades Pesqueras de Provincia de Buenos Aires; ⁵Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP), Paseo Victoria Ocampo n°1, B7602HSA, Mar del Plata, Argentina

ABSTRACT In this work we studied the actual coverage, distribution patterns and ecologic effects of the introduced oyster *Crassostrea gigas* 20 y after their introduction to the Northern Argentinean Patagonia (Bahía Anegada; 39°50'S to 40°40'S and 61°59 to 62°28 W). Using satellite imagery and field and aerial inspections we found 10 oyster beds that cover less than 0.05% of the bay intertidal (area covered: 36.45 ha). These beds are restricted to intertidal zones with superficial hard substrata (limestone outcrops). Most epifaunal organisms (the crabs *Cyrtograpsus angulatus*, *Chasmagnathus granulatus*, the isopod *Melita palmata*, and the snail *Heleobia australis*) showed higher densities inside oyster beds compared with outside and experiments showed that artificially deployed oyster beds increased the densities of their at three intertidal zones (high intertidal marsh, low intertidal marsh, and low intertidal with hard substrata) and also increased densities of infaunal organisms (the polychaetes *Laconereis acuta*, *Nephtys fluviatilis*, and the priapulid *Priapulid tuberculatospinosus*) at the low intertidal with hard substrata. This may be the result of increasing habitat structure and refuge for epifaunal organisms, and enhancement of deposition and sediment stability that may benefit infaunal organisms. Densities bird species (Local species: *Larus dominicanus*, *Haematopus palliatus*; Regional migratory shorebird: *Charadrius falklandicus*; Long range migratory shorebirds: *Pluvialis dominica*, *Calidris canutus*, *Tringa flavipes*) were higher inside oyster beds compared with similar zones without oysters, which may be the result of higher prey availability. Foraging rate was also higher for some of these species (*P. dominica*, *C. falklandicus*). However, due to the limited availability of hard substratum the distribution of oysters is small. In conclusion, no negative effects were observed as a result of this introduction. There was an increase in species abundance and the area was preferred by local and migratory bird species, which also showed higher feeding rates.

KEY WORDS: biologic invasions, *Crassostrea gigas*, ecosystem engineers, migratory shorebirds

INTRODUCTION

The effect of invasive nonindigenous species on native communities had become a major problem in conservation biology (Lodge 1993, Mack et al. 2000, Bax et al. 2001, Byers et al. 2002) mainly because their negative effects on native species, communities and ecosystems (e.g., Vitousek & Walker 1989, Settle & Wilson 1990, Vitousek 1990, Spencer et al. 1991, Carlton 1992, Petren & Case 1996, Juliano 1998, Mack et al. 2000, Byers 1999, Byers 2000, Byers et al. 2002). Marine ecosystems are especially vulnerable to invasive species (Carlton 1996) showing some dramatic effects on biologic diversity and productivity (Bax et al. 2001). However, little attention has been paid to the role of physical ecosystem engineer species (*sensu* Jones et al. 1994) as habitat modifiers when introduced to new habitats. The change of the physical structure of the ecosystem is not currently accounted for in the evaluation of invader's impact (Crooks 2002), but a large effect in the native community is expected when the invader change habitat complexity or heterogeneity (Posey 1988, Crooks & Khim 1999, Crooks 2002, Bruno et al. 2003).

Physical ecosystem engineer species (*sensu* Jones et al. 1994) that create biogenic structure are known to affect community structure (Jones et al. 1997). This effect is because biogenic structure can affect competitive interactions (Fletcher & Underwood 1987), modify the relative importance of predation (Gilinsky 1984, Orth et al. 1984, Dean & Connell 1987, Schwindt et al. 2001, Hamilton

& Nudds 2003) and, ameliorate physical stress (Nixon et al. 1971) and disturbance (Whorff et al. 1995), generally leading to an increase in the density and diversity of associated organisms (Gilinsky 1984, Downes et al. 1998, Zühlke 2001, Bolam & Fernandes 2003, but see Kelaher 2003). Oysters are a good example of substrate and structure creators (see Jones et al. 1997, Gutiérrez et al. 2003), serving as refuge for numerous mobile and sessile species and having a large effect on community structure (Ulanowicz & Tuttle 1992, Kennedy 1996, Dumbauld et al. 2000, Meyer & Townsend 2000, Lenihan et al. 2001) but oysters are also a good example of invasive species in marine and estuarine environments (Carlton 1992, Reise 1998), usually introduced for aquaculture purposes (Carlton 1992, Shatkin et al. 1997, Wasson et al. 2001, Miller et al. 2002). Although ecologic impacts of mollusk farming are known to be small compared with other forms of aquaculture (Naylor et al. 2000), there are several examples of native community modification as a result of *Crassostrea* spp. introduction by direct competition with native species (Shatkin et al. 1997, Reise 1998), by spreading other invasive species (Carlton 1992, de Montaudouin et al. 1999, Byers 1999, 2000, Wolff and Reise 2002), diseases (e.g., Grizel & Héral 1991, Mann et al. 1991, Shatkin et al. 1997, Wolff & Reise 2002), and by decreasing benthic oxygen levels (Castel et al. 1989).

The Pacific Oyster *Crassostrea gigas*, a species endemic from Japan, is today the most successful oyster in commercial cultivation of hatchery-produced seed and is the basis of the largest oyster fisheries in the world (Mann et al. 1991, Reise 1998). *C. gigas* has been extensively introduced outside its native range for culture purposes, and established in the field (Mann et al. 1991, Carlton

*Corresponding author. E-mail: cescapa@mdp.edu.ar

1992, Shatkin et al. 1997). In South America, *C. gigas* was introduced on the Pacific Ocean in Chile and Peru (Winter et al. 1984), and in the Atlantic in Brazil and Argentina (Pascual & Orensanz 1996, Orensanz et al. 2002). In Argentina, this species was illegally introduced during 1982 in Bahía Anegada (39°50'S to 40°40'S and 62°10'W; see Figure 1; Orensanz et al. 2002, Penchaszadeh et al. 2003). A small stock of commercial size of *C. gigas* (ca 500 individuals) imported from Coquimbo (Chile), with gastronomic purposes, were introduced to the southern part of Bahía Anegada with the purpose of implementing an aquaculture production (Orensanz et al. 2002). After a year of experimentation, the remainder small stock was abandoned but they established in the field (Orensanz et al. 2002, Penchaszadeh et al. 2003).

Although there are several conflicts rising from the invasion of *Crassostrea gigas* on northern Patagonia, one important concern is related to conservation of shorebird habitats. The SW Atlantic intertidals are main stopover sites of migratory shorebirds that breed in the northern hemisphere and spend their winter in the southern hemisphere (see Botto et al. 1998). However, the distribution patterns and coverage extension of *C. gigas* on the intertidal and its effects on the benthic community and shorebirds remain unknown. In this context, the main purpose of this research is to evaluate the number, distribution, and extension of *C. gigas* beds today and to evaluate, by sampling and by field experiments, its effects on the benthic community and shorebird habitat use.

MATERIALS AND METHODS

The study was performed in Bahía Anegada (Fig. 1; see Spalleti & Isla 2003 for full description) from December 2001 to November 2003. This is a large embayment (2371 km²) affected by a low amplitude (≤ 1.5 m) semidiurnal tidal regimen.

Given that oysters showed a wide distribution, an analysis of satellite imagery were performed to identify oyster beds and evaluate their spatial distribution. The satellite imagery used was recorded by Enhanced Thematic Mapper plus sensor on board Landsat 7. It records radiation with a nominal spatial resolution of approximately 30 m for bands 1–7, and 15 m for band 8 (panchromatic). Because the tide level is an important factor in saltmarshes and higher levels can mask some habitats, in this study only the image with the lower tide level of a pool of satellite images was selected. The image selected was from 15 January 2002 and was provided by the Argentinean National Commission of Space Activities (CONAE).

To remove the geometric distortions in Landsat imagery, image was geocoded to a UTM Gauss Kruger coordinate system using a first order transformation and nearest neighbor resampling. The root-mean squared error achieved after resampling was lower than 1.5 pixels in all bands. We used map points to geometric corrections. Points were acquired from topographic maps of the Argentinean Army Geographic Institute (IGM; scale 1:50,000).

The south portion of Bahía Anegada was exhaustively searched by walking and the location of oyster beds and different types of

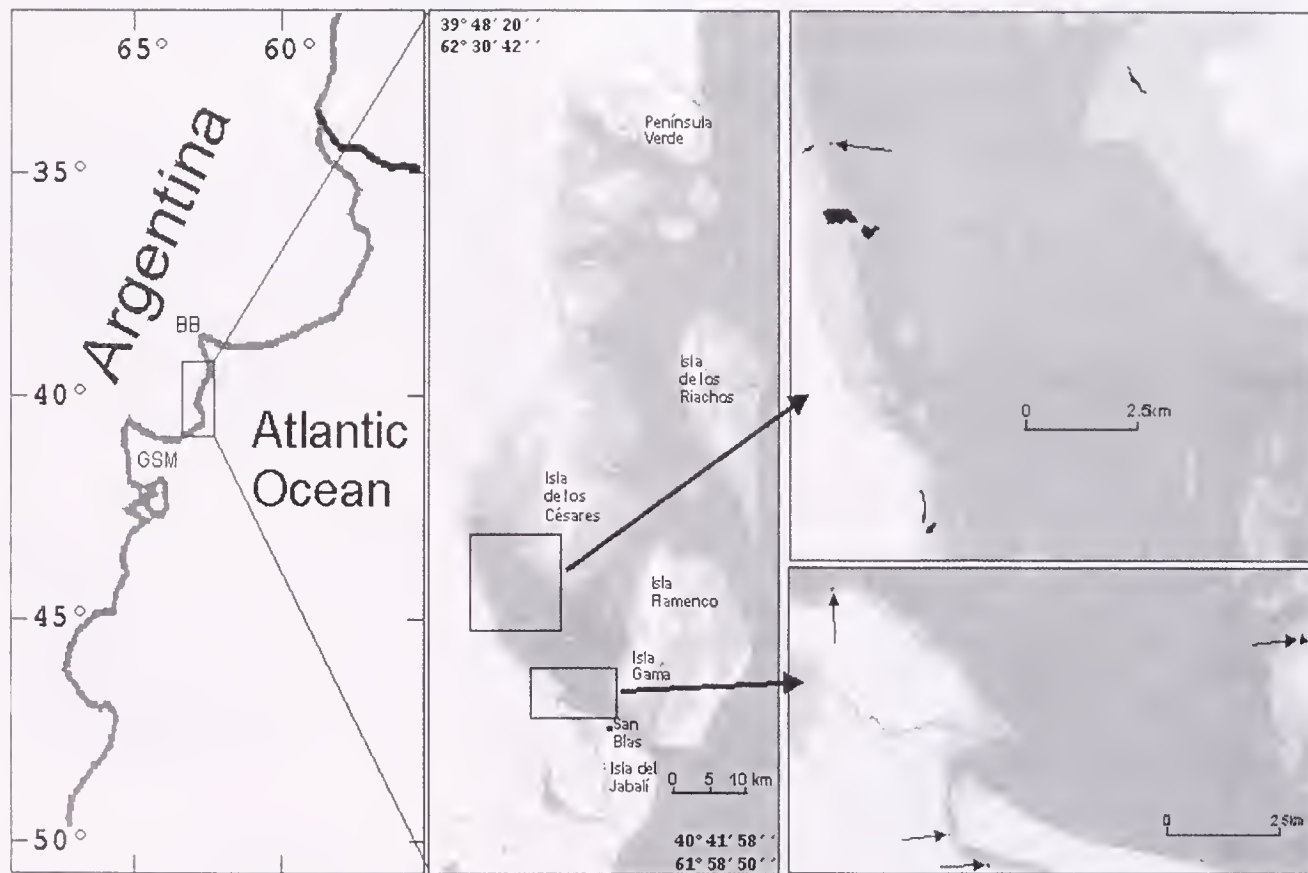


Figure 1. Location and dimension of oyster beds in Bahía Anegada (Argentina). Large oyster beds are shown in black, and smaller ones are indicated by arrows. BB, Bahía Blanca and GSM, Golfo San Matías. Low grey represent intertidal habitat.

environments along the intertidal were registered (position obtained by GPS). A spectral signature analysis of the different kinds of intertidal habitats (tidal flats, saltmarshes dominated by *Spartina alterniflora* Loisel and saltmarshes dominated by *Sarcocornia perennis* (Miller) (formerly *Salicornia ambigua*) were compared with the spectral signature of a known oyster bed. Bahía Anegada intertidal has a mixture of limestone outcrops, sand, silt, and rolling stone bottoms. This variation in substrata, and the variation in water content as a result of field irregularities, generated a bias spectral response that did not allow us to perform a correct classification using common supervised and unsupervised classification methods because of greatly overestimated oyster bed cover. Given this problem, we used a visual analysis of the image, utilizing the Landsat ETM+Panchromatic band (spectral range 520–00 nm), that have a 15 m spatial resolution and allow a good identification of the known oyster beds.

In the Landsat ETM+Panchromatic band, known oyster beds have a particular dendritic pattern that make them conspicuous in the intertidal. This characteristic pattern was used to search for similar structures to make a preliminary oyster beds map. Then, an exhaustive aerial inspection (flying between 60 and 100 m altitude and between 100 and 150 km per hour) of Bahía Anegada coast and islands was done during low tide to verify the presence of oyster beds. No new oyster beds were found but some structures previously identified as oyster beds were discarded. Discrimination of land, intertidal and water was done using the Idrisi32 Maximum Likelihood Software module. The final map (Fig. 1) was done by adding the identified oyster beds to the thematic map.

To study the distribution patterns in relation to intertidal level, transects (100 m) perpendicular to the shoreline were performed (spaced by at least 50 m). In each transect, the number of oysters in 1 m² were counted every 0.4 m of intertidal height. The null hypothesis of no differences in oyster densities between tidal levels was analyzed with 1-way ANOVA (Zar 1999). To evaluate the type of substrate used by oysters, the depth at which the hard substratum (i.e., limestone outcrops) was located was measured by pushing a 1.5 m iron stick and noting the buried length of the stick when it touch the hard substratum, and the number of oysters (m⁻²) were counted. The null hypothesis of no relation between hard substratum depth and oyster densities was analyzed with correlation analysis (Zar 1999).

To evaluate whether there is a relation between oyster beds and densities of epifaunal organisms, samples of 0.25 × 0.25 m were assigned inside and outside oyster beds. In each sample, epifaunal organisms were counted and identified to the lowest taxonomic level possible. For each taxa, the null hypothesis of no differences in densities of organisms were analyzed with *t*-test (or Mann-Whitney when necessary, Zar 1999). To evaluate if there was any relation between oyster densities and the densities of the predominant native grapsid crab *Cyrtograpsus angulatus* Dana, a species that is known to be strongly and positively affected by biogenic structure (see Schwindt & Iribarne 2000, Schwindt et al. 2001, Mendez Casariego et al. 2004), a different sample design was used. The number of oysters and crabs were counted in 83 sampling units of 1 m², randomly chosen along the intertidal. The null hypothesis of no relation between densities of oysters and crabs were evaluated with correlation analysis. Similarly, to evaluate the relation between oyster beds and densities of the snail *Helicobia australis*, the intertidal substrate was divided into 3 zones (High = 1.3 m above the lower tidal level (ALTL), medium = 0.7 m ALTL and low = 0.2 m ALTL). In each zone, the number of snails in 25

samples of 0.2 × 0.2 m was counted in pools inside and outside oyster beds. For each intertidal level, the null hypothesis of no differences in snail densities between sites was evaluated with *t*-test (Zar 1999).

To experimentally evaluate the effect of *Crassostrea gigas* on natural community on the high marsh, the low marsh and the low intertidal with hard substrata, 60 square plots (1 m²), 20 in each zone, were randomly chosen on January 2002. Each plot was assigned to one of the following treatments; (1) artificial oyster bed or (2) control. Artificial oyster bed treatments were done by transplanting oysters from natural oyster beds to the plots. These oysters were washed with seawater to eliminate epifauna before transplanting. After 11 mo, epifaunal organisms were counted and classified on a 0.25 × 0.25 m square from the center of the plot. In each plot, a core sample (35-cm depth, 15-cm diameter) was also taken. Infaunal organisms were separated by sieving the samples through a 0.5-mm screen. Organisms were counted and classified to the lowest taxonomic level possible. The null hypotheses of no differences in densities of organisms of each species between treatments (with and without oysters) for each intertidal zone were analyzed with *t*-test or nonparametric Mann-Whitney test (Zar 1999).

One of the main concerns in the region came from the potential effect of oyster beds on habitat use by birds, mainly neotropical migratory shorebirds. To evaluate differential use of areas by shorebirds, 3 sites were selected at the SW part of the bay (Isla Jabali, Fig. 1 lower right) each one had oyster reefs and nearby areas without oysters. Each bed had a mean density of 47.4 oysters m² (SD = 12.3) and a mean surface area of 1 ha (Borges 2002, Penchaszadeth et al. 2003). Areas were selected keeping similar characteristics such as tidal level, slope and compass orientation (NW-SE) and known to be used by shorebirds. In each area, a telescope (18 × 36) was used to perform censuses from December 2001 to April 2002 (this period represent the entire migratory season). In each census, individuals were identified and the percentage of them feeding in each area was calculated. Abundance of each shorebird species was compared between sites and months with repeated measures ANOVA (Neter et al. 1990). To evaluate whether there is a relationship between the oyster beds and the shorebird foraging rate and efficiency, focal observations (using a 18 × 36 telescope) were performed. Each bird was observed for a period between 5 and 10 min. Before the observations, and based on a previous sampling, a list of all acts to be recorded was defined (walk, pause, peck, and capture of item) and maintained during the study to ensure standardized observations. The number of probes per minute and the proportion of probes resulting in successful prey capture were calculated. Differences in rate of consumption and foraging efficiency between areas were evaluated independently for each bird species with *t*-test (Zar 1999).

RESULTS

A total of 10 oyster beds were detected, 3 of small size (size range 0.09–0.36 ha), 6 of medium size (size range 1.62–5.67 ha) and 1 large bed (size 16.38 ha), all of them located in the southern part of the bay (Fig. 1). Oyster beds cover a total of 36.45 ha, which is less than 0.05% of the Bahía Anegada intertidal (total intertidal area = 89,689 ha). Both aerial and walk inspection reveals that besides the areas colonized by oysters there were no free superficial hard substrata along the intertidal.

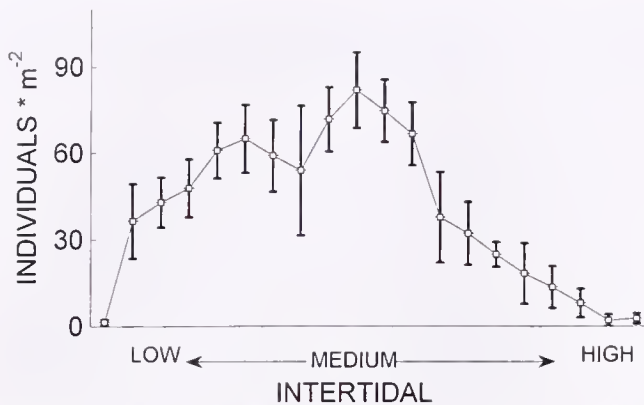


Figure 2. Oyster densities across the intertidal. Squares represent means and bars standard deviation.

Spat were found attached to limestone outcrops, empty shells, shells of a native small mussel *Brachidontes rodriguezi* (d'Orbigny) and the basal portion of *Spartina alterniflora*. Oyster densities were higher in the middle and middle to low intertidal compared with other intertidal zones (ANOVA, $F_{19,177} = 56.404$, $P < 0.001$, Fig. 2). Moreover, there were no oysters in the low intertidal or in the subtidal. There was a negative correlation between hard substratum (i.e., limestone) depth and oyster densities ($r^2 = 0.597$, $n = 100$, $P < 0.001$) and there were no oysters in zones with hard substratum depths higher than 10 cm.

Inside the oyster matrix, the number of juveniles of the crab species *Chasmagnathus granulatus* Dana and *C. angulatus*, and the number of the polychaete *Laconereis acuta* (Treadwell) and the isopod *Melita palmata* (Montagu) were higher inside oyster beds but the number of the hermit crab *Pagurus criniticornis* (Dana), the snail *Heleobia australis* (d'Orbigny), and unidentified anemones were higher outside oyster beds (Table 1). There were no differences in number of the small mussels *Brachidontes rodriguezi* (d'Orbigny) (Table 1).

There was a positive correlation between oyster density and the density of the crab *Cyrtograpsus angulatus* ($r^2 = 0.27$, $df = 43$, $P < 0.001$). Similarly, densities of the snail *Heleobia australis* were higher in pools inside oyster beds along the 3 intertidal heights compared with pools outside oyster beds (high: $t = 9.79$, $df = 48$, $P < 0.001$; medium: $t = 3.402$, $df = 48$, $P < 0.005$; low: $t = 2.49$, $df = 48$, $P < 0.05$; Fig. 3).

In the high marsh, the number of the small mussels (square root transformed data; $t = 8.63$, $df = 18$, $P < 0.001$), anemones (log

transformed data; $t = 2.38$, $df = 18$, $P < 0.05$) and the crab *Cyrtograpsus angulatus* (log transformed data; $t = 7.32$, $df = 18$, $P < 0.01$) were higher in artificial oyster beds plots compared with control plots. The amphipods *Corophium* sp. and crabs of the species *Chasmagnathus granulatus* were only present in the artificial oyster bed plots. In contrast, the number of snails (*Heleobia australis*) was higher in the control plots ($Z = 2.86$, $N_1 = N_2 = 10$, $P < 0.005$). In the low marsh, there were no differences in the number of mussels between treatments ($t = 0.45$, $df = 18$, $P > 0.5$). Anemones, amphipods, polychaetes and, the crabs *C. angulatus*, *C. altimanus* Rathbun and *C. granulatus*, were present only in the artificial oyster bed plots. *H. australis* was present only in control plots. In the low intertidal, mussels and the crab *C. angulatus* were present only in artificial oyster bed plots. Inside the sediment, there were no differences in the number of mussels ($Z = 1.51$, $P > 0.1$), polychaetes of the species *Laconereis acuta* ($Z = 1.57$, $P > 0.1$) and *Neptilyx fluviatilis* Monro ($Z = 0.1$, $P > 0.5$), and the priapulid *Priapululus tuberculatospinosus* Baird ($Z = 0.1$, $P > 0.5$) between plots in the high marsh. In the low marsh, the number of polychaetes (*L. acuta*) were higher in control plots compared with artificial oyster bed plots ($Z = 1.256$, $N_1 = N_2 = 10$, $P < 0.05$) but there were no differences in the number of mussels between plots ($X_1 = 22.8$, $SD = 8.13$, $X_2 = 22.4$, $SD = 11.8$; $t = 0.09$, $df = 18$, $P > 0.9$).

The birds present during the study period were the Two Banded Plover *Charadrius falklandicus* (Latham), the American Golden Plover *Pluvialis dominica* (Müller), the Red Knot *Calidris canutus* (Linnaeus), the Lesser Yellowlegs *Tringa flavipes* (Gmelin), the American Oystercatcher *Haematopus palliatus* Temminck, and the Kelp Gull *Larus dominicanus* (Lichtenstein). For all these species (Fig. 4), densities inside oyster beds were higher than in the adjacent areas without oysters (American Oystercatcher: $t = 2.955$, $df = 60$, $P < 0.005$, the Two Banded Plover: $t = 5.772$, $df = 60$, $P < 0.001$, the American Golden Plover: log transformed data, $t = 12.667$, $df = 60$, $P < 0.001$) and the Kelp Gull: log transformed data, $t = 4.158$, $df = 60$, $P < 0.001$). The Red-knot ($X = 0.024$ ind* m^2 , $SD = 0.0199$) and the Lesser Yellowlegs ($X = 0.0023$, $SD = 0.0049$) were only present inside oyster beds. Foraging rate (Fig. 5) was higher inside oyster beds for the American Golden Plover ($t = 2.172$, $df = 31$, $P < 0.05$) and for the Two Banded Plover ($t = 2.294$, $df = 15$, $P < 0.05$) but there was no difference for the Oystercatcher (Mann-Whitney U -test, $Z_{adj} = 0.759$, $N_1 = 10$, $N_2 = 6$, $P > 0.1$) nor for the Red-Knot ($Z_{adj} = 0.039$, $N_1 = 17$, $N_2 = 5$, $P > 0.5$). There were no differences in foraging efficiency between sites (American Golden Plover: $t = 0.714$, $df = 31$, $P > 0.1$; Two Banded Plover: $t = 0.864$, $df = 15$, $P > 0.1$; Red Knot: $t = 0.857$,

TABLE 1.

Densities of species of epifauna inside and outside oyster beds. Density was expressed as individuals per sample unit (0.0625 m^2).

	Inside Oyster Beds	Outside Oyster Beds	d.f.	T or Z Value	P	Observations
<i>Chasmagnathus granulatus</i>	18.67 (7.75)	4.07 (5.35)	28	6.003	<0.001	t-test
<i>Cyrtograpsus angulatus</i>	3.2 (2.14)	0.4 (0.74)	28	5.065	<0.001	t-test, log
<i>Priapululus tuberculatospinosus</i>	0.26 (0.39)	1.24 (0.47)	28	6.26	<0.001	t-test, log
<i>Brachidontes rodriguezi</i>	0.2 (0.77)	1.13 (2.7)	28	1.44	>0.1	M-W
<i>Heleobia australis</i>	3.4 (8.75)	13.07 (10.74)	28	3.038	<0.005	M-W
<i>Laconereis acuta</i>	1.73 (1.33)	0.67 (0.9)	28	2.19	<0.05	M-W
<i>Melita palmata</i>	1.07 (1.94)	0.13 (0.35)	28	0.79	>0.1	M-W
Unidentified Cnidaria	0.47 (0.52)	3.07 (2.66)	28	2.51	<0.05	M-W

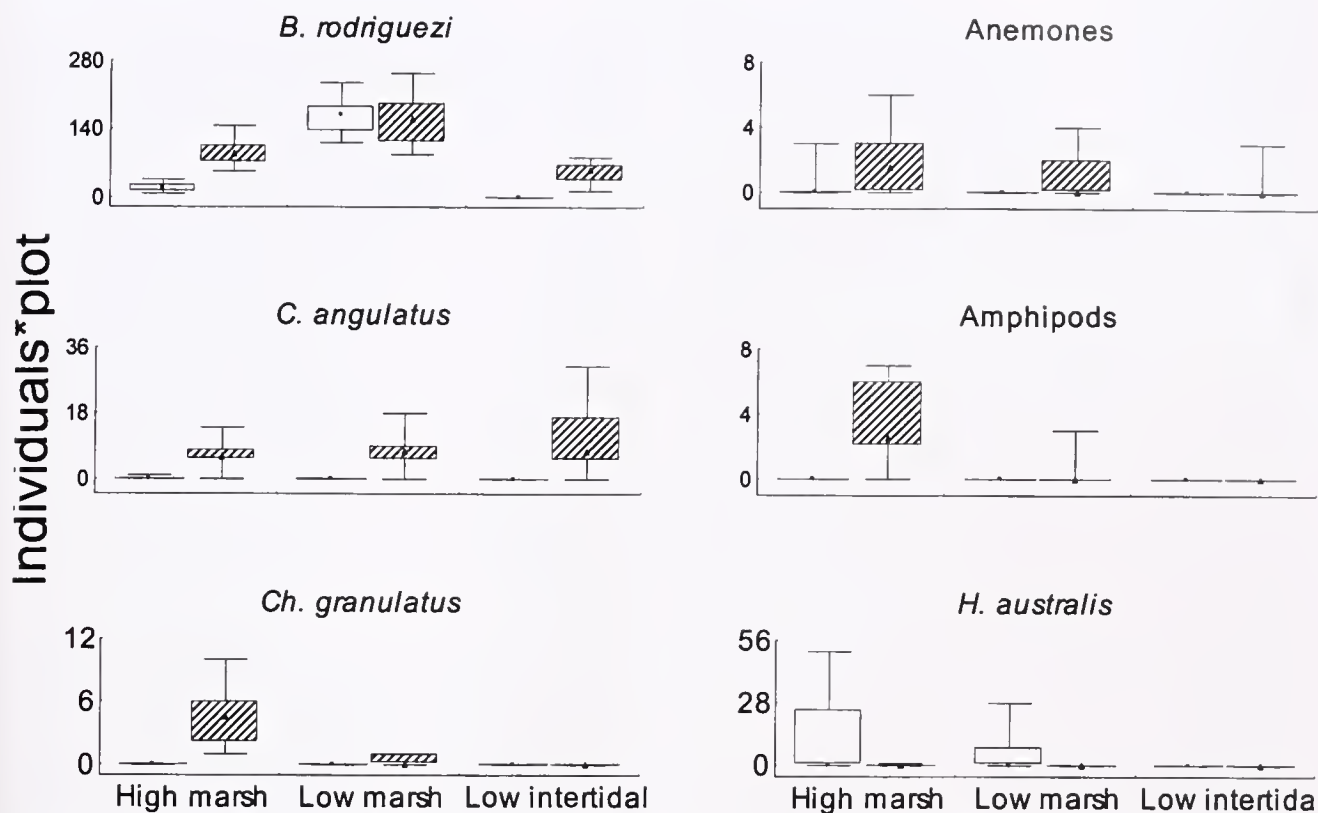


Figure 3. Number of individuals of epifaunal species in control plots (1 m²) (empty boxes) and artificial oyster bed plots (dashed boxes) at three zones of the intertidal. Here and thereafter squares and triangles inside boxes represents medians, limits of boxes are 25th and 75th percentiles, lines represents 1st and 99th percentiles.

df = 20, $P > 0.1$; American Oystercatcher; $t = 0.402$, df = 14, $P > 0.5$).

DISCUSSION

After more than 20 y of introduction, the Pacific oyster *Crassostrea gigas* has established in Bahía Anegada but covers a very small percentage ($\leq 0.05\%$) of their intertidal area. This contrasts with previous introductions of *C. gigas* around the world (and in wide array of environmental conditions), where *C. gigas* populations expanded in relatively short time (see Shatkin et al. 1997, Sumner 1980, Reise 1998). However, our evidences shows that in this area the distribution is limited by the lack of hard substratum, which is the result of a large sediment discharge from the Colorado River (see Spalletti & Isla 2003 for details). Although this river does not discharge now into the bay, the muddy sediments are still the dominant feature of this area.

We found oysters only in the intertidal zone with higher densities at the middle intertidal. This result contrasts from the pattern found in other sites, where densities of this species are higher in the low intertidal (Reise 1998). This pattern can result from the distribution of hard substrata (limestone outcrops). Oysters were present only in zones with superficial hard substrata; soft bottoms cannot support oysters on the surface (Reise 1998). The local oyster *Ostreola spreta* (d'Orbigny) (formerly known as *Ostrea spreta*) also settle on any hard surface (de Castellanos & Cabrera 1957, de Castellanos 1968), and is the dominant settling species in artificial collection of seed oysters deployed in this area (Borges et al. 2002). However, survival at the intertidal is low probably due low tolerance to higher temperatures (Stenzel 1971), even when

clusters of *C. gigas* offer a good settlement site (Penchazadeth et al. 2003). We have no information on the interaction between the two oysters, but the distribution pattern may in part be the results of competition. Thus, any effects of *C. gigas* on the local community are restricted to zones with superficial hard substrata (mainly limestone outcrop) in the middle intertidal.

Densities of the crabs *Cyrtograpsus angulatus* and *Chasmagnathus granulatus*, and of the amphipod *Corophium* sp. and the isopod *Melita palmata* were higher inside oyster beds and, as expected, experiments indicated densities of epifaunal organisms were higher in oyster beds than outside of the beds. The importance of availability of shelter and structure complexity on inter and subtidal community structure has long been recognized (e.g., Barshaw & Lavalli 1988, Fernández et al. 1993a, Fernández et al. 1993b, Gee & Warwick 1994, Moksnes et al. 1998, Lohrer et al. 2000, Robinson & Tully 2000, Jensen et al. 2002). Oyster shells are an important shelter creator with dramatic effects on crab densities (see Wainwright et al. 1992, Iribarne et al. 1995). The relationship between *C. angulatus* and habitat structure created by an introduced species has also been found in other SW Atlantic estuary (i.e., Mar Chiquita coastal lagoon) invaded by the reef building polychaete *Ficopomatus enigmaticus* Faubel (Schwindt & Iribarne 1999, Schwindt et al. 2001). In this case, oyster shells can increase crabs density not only by enhancing recruitment (see Fernández et al. 1993b) but also by decreasing adult predation risk (Mendez Casariego et al. 2004). In contrast, densities of the snail *Heleobia australis* and the hermit crab *Pagurus criniticornis* were lower inside oyster bed matrix. This can be because, for these two species, availability of shelter may not be a limiting factor (both

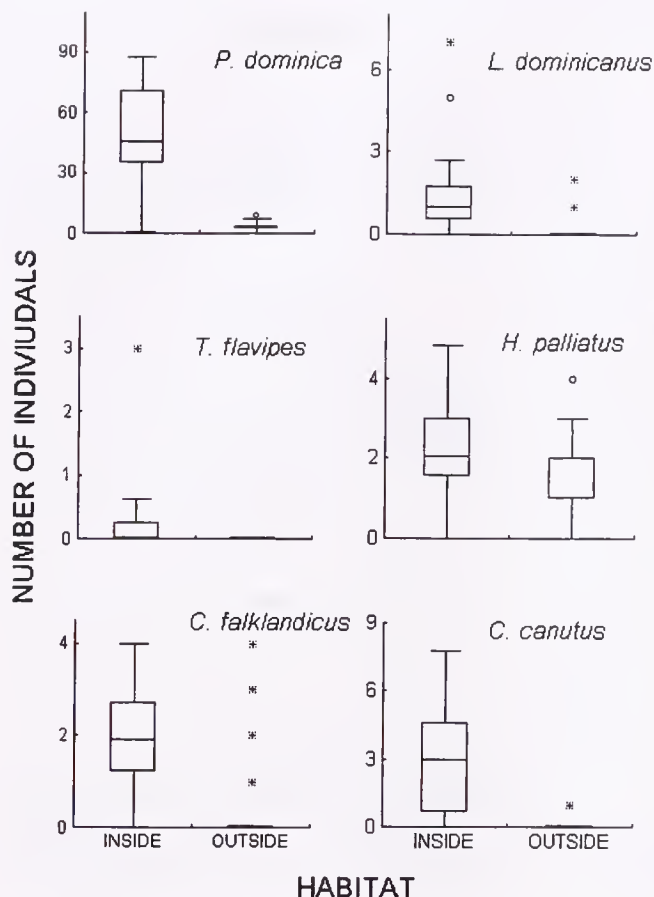


Figure 4. Average counts of number of individuals of different bird species using inside and outside oyster bed habitats. Circles represent outliers and asterisks are extremes.

species carried their own refuge) and they may be negatively affected by 3-dimension structure (see Kelaher 2003). Alternatively, predation risk may increase inside the oyster matrix because the higher abundance of predators such as crabs. Indeed, *H. australis* shells are usually found in *C. angulatus* stomach contents (P. Martinetto, pers. com.).

The increase of epifauna inside oyster bed plots may be the result of immigration, different mortality and recruitment (Crooks

& Khim 1999). Individuals of *C. granulatus* that occur in oyster bed plots were only adults suggesting colonization but not recruitment, in contrast, there were recruit and small juveniles of *C. angulatus* and all these stages plus adults of *C. altimanus* suggesting recruitment and colonization.

Densities and foraging rates of shorebirds were higher inside oyster beds and foraging efficiencies, whereas foraging inside or outside oyster beds, were the same. Wolff & Reise (2002) linked the decreases of the oystercatcher *Haematopus ostralegus* Linnaeus populations in the European Wadden Sea, with the introduction of *C. gigas*, and posterior transformation of mussel beds of *Mytilus edulis* d'Orbigny into oyster beds. In addition, bird foraging efficiency, while preying on intertidal invertebrates, are negatively affected by substrate heterogeneity (e.g., Common eiders on Rockweed beds; Hamilton & Nudds 2003; also Marsh 1986). Nevertheless, our results shows that prey abundance (particularly crabs and snails) were positively affected by the presence of biogenic structure created by oysters. Thus, as in mussel cultures (Caldow et al. 2003), higher prey abundance inside oyster beds may be influencing bird habitat choice by positively affecting their foraging rate.

Studies on the effect of naturalized pacific oyster beds on benthic communities also showed strong effects by decreasing O_2 on sediments as a result of organic matter enrichment (Castel et al. 1989, Nugues et al. 1996), by outcompeting local bivalve species (Shatkin et al. 1997, Reise 1998) and, by creating physical structure on otherwise flat zones (Castel et al. 1989). At Bahía Anegada the vascular plant *Spartina alterniflora* and the clumps of the small mussel *Brachydontes rodriguezi* offer settlement sites for this oyster (Borges 2001, Penchaszadeth et al. 2003). Interestingly, oysters and mussels can generate layers of settlement, forming clusters that sometimes can be used as settlement sites by *Ostreola spreta* (Borges 2001, Penchaszadeth et al. 2003). These changes in the physical structure of the ecosystem itself, was postulated as one form in which invaders can have strong ecosystem-level effects (Bertness 1984, Chapin et al. 1997, Crooks & Khim 1999, Crooks 2002) and is supported by some examples (e.g., Posey 1988; see also Crooks 2002 for a review). In contrast, other studies show an increase on macrofaunal abundance as a result of refuge creation (De Grave et al. 1998).

In summary, based in our observations on the distribution and coverage of the reefs of *C. gigas* and the expansion rates reported for other areas worldwide, the success of this oyster is low. However, in terms of the potential expansion, our result may be misleading, given that the distribution is controlled by the availability of substratum.

ACKNOWLEDGMENTS

The authors thank to Mr. M. Pereda for field help, Mr. A. Alzugaray (Municipality representative at San Blas) for general assistance, Dr. F. Isla for lending us satellite image processing software Idrisi 32, Dr. E. Spivak for helping with taxonomy and the Argentinean National Commission of Space Activities (CONAE) for providing us satellite images. This project was partially supported by Universidad Nacional de Mar del Plata, Fundación Antorchas (Argentina A013672 and 13900-13), National Geographic Exploration Grants (#6487-99), ANPCyT (1-7213) and CONICET (PIP 2851, all granted to O.I.). M. Escapa, P. Daleo, J. Alberti were supported by Doctoral scholarships from CONICET.

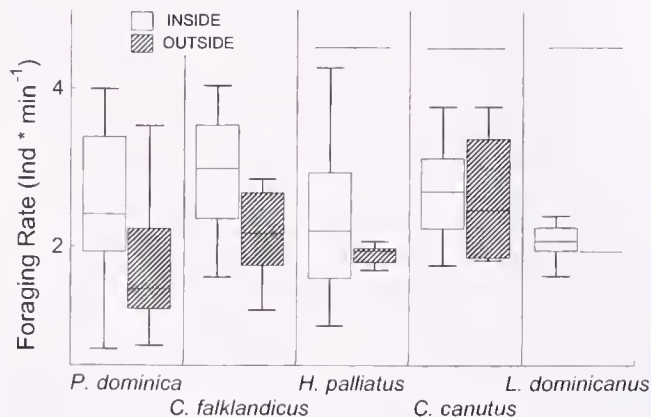


Figure 5. Foraging rate of different bird species compared between inside (empty boxes) and outside (dashed boxes) oyster shell habitats.

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THE POTENTIAL OF AERIAL PHOTOGRAPHY FOR ESTIMATING SURFACE AREAS OF INTERTIDAL PACIFIC OYSTER BEDS (*CRASSOSTREA GIGAS*)

B. J. KATER* AND J. M. D. D. BAARS

Netherlands Institute for Fisheries Research, Centre for Shellfish Research, P.O. Box 77, 4400 AB, Yerseke, The Netherlands

ABSTRACT Pacific oysters were introduced into the Eastern Scheldt in 1964 for breeding purposes. The first spatfall of wild Pacific oysters was recorded in 1976, and a second larval outburst in 1982 definitely settled wild Pacific oysters in the Eastern Scheldt waters. Oyster beds on intertidal and subtidal areas have been growing since. The objective of this study is to research the potential of aerial photography for estimating surface areas of intertidal Pacific oyster beds. Black & white and false-color aerial photographs were used to locate Pacific oyster beds. For verification purposes, oyster bed contours were measured in the field. The accuracy of the method used was comparable with accuracies found in other studies, with a chance of underestimating the surface areas in the field. With aerial photographs of 1980 and 1990 the surface areas of Pacific oysters in both years were reconstructed, showing an increase in surface area of Pacific oyster beds. The study shows aerial photography has the potential to be an aid in surveying intertidal Pacific oyster beds.

KEY WORDS: aerial photography, *Crassostrea gigas*, reconstruction, Eastern Scheldt

INTRODUCTION

In the Eastern Scheldt Estuary (The Netherlands) flat oysters (*Ostrea edulis*) have been cultured since 1875 (Shatkin et al. 1997). The estimated stock size in 1962 was 120 million. After the severe winter of 1962/1963, which caused high mortality, the stock was reduced to 4 million oysters (Drinkwaard 1999). Searching for alternatives, Dutch oyster farmers introduced the Pacific oyster to the Eastern Scheldt in 1964. The Pacific oyster *Crassostrea gigas* (Thurnberg 1793) is native to Japan. The species has been introduced in several other areas, e.g. Australia, New Zealand, France, United Kingdom, Ireland and United States (Shatkin et al. 1997). The reasons for introduction include the economic pressures in the presence of diminishing wild fisheries resources, destruction of a fishery because of disease, or nonexistence of native fishery (Mann 1979).

The introduction of Pacific oysters in the Eastern Scheldt was found acceptable for 2 reasons. The first reason was the expected closure of the Eastern Scheldt with a dam, changing the Eastern Scheldt into a stagnant lake (Nienhuis & Smaal 1994) in which Pacific oysters would not survive. But in 1976 the Dutch government decided to build a storm surge barrier instead of a dam, leaving the Eastern Scheldt a tidal ecosystem (Nienhuis & Smaal 1994). The second reason was that offspring were not to be expected at the Eastern Scheldt latitude (Drinkwaard 1999). However, first spatfall on dike foos and jetties were recorded in 1976, and political pressure stopped importation of Pacific oysters in the following year. A second larval outburst in 1982 definitely settled wild Pacific oysters in the Eastern Scheldt waters (Drinkwaard 1999), and oyster beds on intertidal and subtidal areas have been growing since.

One of the main problems in the Eastern Scheldt concerning expansion of Pacific oysters is the potential interaction with commercially exploited species like cockles (*Cerastoderma edule*), blue mussels (*Mytilus edulis*), and cultivated oysters (*Crassostrea gigas*). Wild Pacific oysters can compete with these commercial species for food and space (Smaal et al. 2001). Although the growth of Pacific oyster distribution has been recognized as a possible problem, surveying oyster beds only started in 1999. To

reconstruct former relations between commercial shellfish species and expansion of the Pacific oysters, reconstruction of former surfaces with oyster beds is necessary. Remote sensing techniques like aerial photography can be helpful in this reconstruction. Aerial photography is often used as a tool for surveying e.g. estimating the population size of breeding Greylag Geese (Kristiansen 1997), green algal mats in the Skagerrak (Pihl et al. 1999), vegetation mapping in a tropical freshwater swamp in Australia (Harvey & Hill, 2001), seagrass coverage in Australia (Kendrick et al. 2002), and detection of dead or defoliated spruces in Finland (Haara & Nevalainen 2002).

The first objective of this study is to research the potential of aerial photography for estimating surface areas of intertidal Pacific oyster beds: how well can Pacific oyster beds be seen and distinguished from other structures on aerial photographs? The second objective is to reconstruct the surface areas of intertidal Pacific oysters from the past.

MATERIALS AND METHODS

Oyster Beds on Aerial Photographs

This study was performed on intertidal areas in the Eastern Scheldt (The Netherlands). Figure 1 shows the location of the study area.

Hardcopies of black & white photographs and false-color slides, made disposable by the National Institute for Coastal and Marine Management/RIKZ, were used in this study. Most photographs were taken on a scale 1:10000, but sometimes a scale of 1:5000 (Verdronken Land van Zuid-Beveland, 1980) or 1:20000 (Roggenplaat 1991 and 1999) was used.

The first reconstruction period is dated between the first and second Pacific oyster larval outbursts in the Eastern Scheldt. Most available photographs dated from 1980, so this year was chosen for reconstruction. All photographs were black and white. The second reconstruction period is dated after the second larval outburst of 1982. Based on photo availability, 1990 was chosen as the year to be reconstructed, and areas not photographed in 1990 were completed using photographs from 1989 and 1991. The last reconstruction dealt with the present situation—photographs taken in 1999 and 2000 were used.

Aerial photographs were digitalized for computer-aided analy-

*Corresponding author. E-mail: b.j.kater@rikz.rws.minvenw.nl

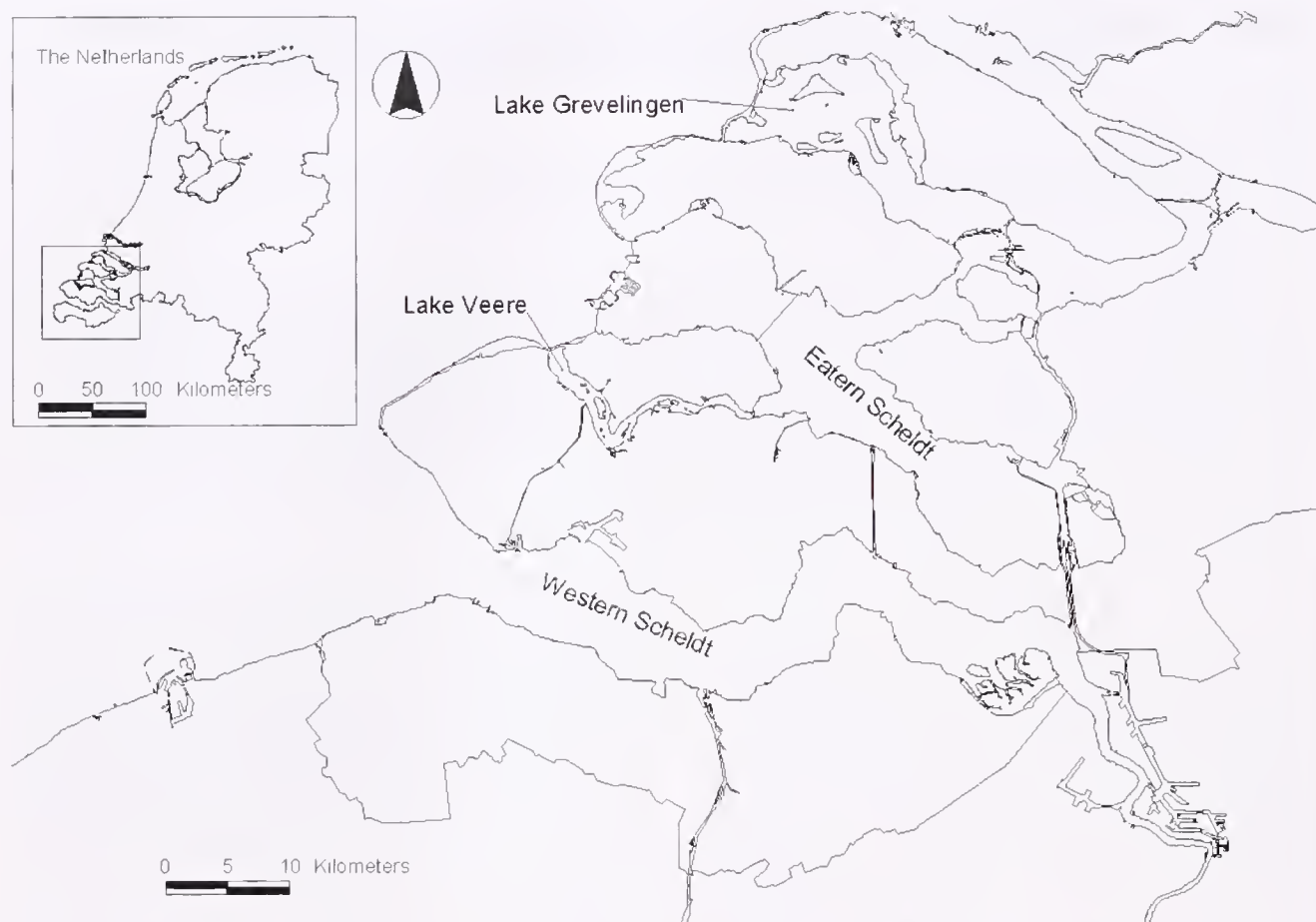


Figure 1. Map of the Netherlands, showing the location of the Eastern Scheldt.

sis. Each photograph was scanned (Microtek Scanmaker 9600 XL). A resolution of 200 dots per inch was used, achieving the desired clarity together with minimal storage space.

All photographs were georeferenced to a map of the Eastern Scheldt, using the extension World File Creator (McVay 1998). Georeferenced photographs were brought into a Geographic Information System (GIS) using ArcView 3.2a.

Oyster beds appear as dark spots on both black & white and false-color photographs. On each photograph oyster beds were located by eye, and within ArcView a polygon was drawn around each bed. The surfaces of each bed were calculated using the extension XTOOLS (DeLaune 2001).

Groundtruth

For verification purposes, oyster bed contours were measured in the field. At low tide, oyster beds lying in an intertidal area fall dry. Contours of these oyster beds were measured by walking around them with a Global Position System (Garmin 12). At each change of direction a waypoint was marked. Waypoints were transferred to ArcView, and waypoints of each oyster bed were connected to form one polygon. Contours were measured in January and February of the years 2000, 2001, and 2002.

Verification Techniques

To compare results obtained with aerial photography, 3 locations were selected based on available information from aerial

photographs and field surveys: Zandkreek, Slikken van Viane, and Stavenisse (Fig. 2).

With the extension XTOOLS (DeLaune 2001) the surface areas of oyster beds, derived from the field survey and aerial photographs of each location, were calculated. These surface areas were used to get an impression of errors introduced by estimating areas from aerial photographs. Two types of errors can be made. First, (parts of) oyster beds seen on an aerial photograph but not found in the field, are called false positives. Second, (parts of) oyster beds not seen on an aerial photograph, but located in the field, are called false negatives.

For further verification purposes, in a GIS a grid of 5 m × 5 m was laid on the intertidal area (i.e., where oysters could be located) of the three chosen locations. To each grid cell the following information was added: (a) are there oyster beds seen during the ground truth present in the grid cell and (b) are there reconstructed oyster beds present in the grid cell. If a grid cell contained both ground truth beds and reconstructed beds, or no ground truth beds and reconstructed beds, it was labeled as correct.

First, the overall accuracy was calculated by dividing the sum of correctly labeled grid cells by the total number of grid cells (Congalton 1991). This is a reasonable way to describe the overall accuracy of a map, but does not account for the component of accuracy resulting from chance alone (Mumby et al. 1997).

Secondly, the user's accuracy (or reliability) was calculated. This measure is indicative of the probability that a grid classified



Figure 2. Map showing areas used for verification purposes.

on the image is actually there on the ground (Story & Congalton 1986). The user's accuracy is calculated by dividing the total number of grid cells that were correctly classified in a category (in this study there are 2 categories: oysters or not-oysters) to the total number of grid cells that were classified in that same category (Congalton 1991).

Finally, a τ coefficient was calculated to give an indication of the number of grid cells classified correctly excluding the correct classification on chance alone (Ma & Redmond 1995). This coefficient is described by:

$$T = \frac{P_0 - P_r}{1 - P_r}, \text{ where } P_r = \frac{1}{N^2} \sum_{i=1}^2 n_i * x_i$$

where

P_0 : overall accuracy

n_i : Total number of grid cells in oyster beds and outside oyster beds

x_i : Total number of correctly classified grid cells in oyster beds and outside oyster beds

N : Total number of grid cells

Construction of an Up-to-Date Oyster Map

To construct an oyster map that describes oyster beds as up to date as possible, all contours measured in 2002 were mapped. Contours measured in 2000 and 2001 were added to this map, but only in areas not described by the 2002 contours. Next, the contours obtained from aerial photographs taken in 1999 and 2000 were added to the map, but only of beds of which no contours were measured. Finally, all areas not described by one of the measured techniques, were described using the reconstruction of 1990. Total surface areas of intertidal oyster beds were estimated using the ArcView extension XTOOLS (DeLaune 2001).

RESULTS

Reconstructions

Aerial photographs taken in 1980 covered about 60% of the intertidal area in the Eastern Scheldt. In particular, areas in the southwestern part are missing. The surface area of oyster beds reconstructed in the photographed area is 15 ha. The total surface area of oyster beds can be estimated by correcting for the areas not

photographed, assuming a homogeneous scattering of oyster beds. The total estimated surface in 1980 is 25 ha.

The year 1990 had the largest coverage of the intertidal area with aerial photographs; photographs cover 89% of the intertidal area in the Eastern Scheldt. Figure 3 shows the results of the reconstruction of oyster beds in 1990. The surface area of reconstructed oyster beds in photographed areas is 210 ha. The corrected estimated surface area is 236 ha.

Available aerial photographs in 1999 and 2000 covered about 40% of the intertidal areas of the Eastern Scheldt.

False Positives and False Negatives

The surface areas of oyster beds found in field surveys and on aerial photographs, and the surface areas of false positives and false negatives, are shown in Table 1. On Stavenisse no false positives (i.e., oyster beds seen on aerial photographs, but not in the field situation) were found. At the other two locations the percentages of false positives were low (2% to 6%). The average percentage of false positives was 2.4%. In all three locations a high percentage of false negatives (no oyster beds seen on aerial photographs, but nonetheless found in the field situation) were found (41% to 59%). The average percentage of false negatives was 53%.

TABLE 1.

Surface areas of oyster beds in field (ha) and on aerial photographs (ha), and surface areas of false positives (ha) and false negatives (ha), for three locations in the Eastern Scheldt.

Location	Field	Photo	False Positive	False Negative
Slikken van Viane	27	17	1.5	11
Zandkreek	73	33	1.6	42
Stavenisse	16	7.0	0	9.5

Accuracy

The overall accuracy, user's accuracy of oysters and tau accuracies of the three selected locations are presented in Table 2. The table shows a high average overall accuracy of 87%, a user's accuracy of 74% and a tau accuracy of 61%.

Oyster Map 2002

The oyster map 2002 is made up of contours measured in 2002, 2001, and 2000, and aerial photographs of 2000 and 1990. Table 3 gives an overview of the contribution of field survey and aerial photography to the 2002 map. The table shows more than 60% of



Figure 3. Reconstruction of oyster beds in 1990.

TABLE 2.
Verification.

	Overall Accuracy	User Accuracy	Tau Accuracy
Zandkreek	84%	60%	58%
Sint Annaland	82%	84%	64%
Viane	94%	77%	62%
Average	87%	74%	61%

the oyster map is based on contours measured in the field. The total surface area of the Eastern Scheldt covered with oyster beds is estimated 640 ha. Figure 4 shows the positions of oyster beds.

DISCUSSION AND CONCLUSIONS

This study describes the usage of aerial photographs to reconstruct the surface areas of intertidal beds of Pacific oysters in the Eastern Scheldt. Using aerial photography for the assessment of

TABLE 3.
Contribution of field survey and aerial photographs to the estimation of total area of oyster beds in 2002.

	Contribution (%)
Contours measured in 2002	37%
Contours measured in 2001	5%
Contours measured in 2000	19%
Aerial photographs 1999–2000	27%
Aerial photographs 1990	12%

} 61%

shellfish stocks is not often described. Most studies focus on seagrass beds (e.g., Bulthuis 1995, Pasqualini et al. 1998, Jaubert et al. 1999). Reconstruction of situations in the past has been done before, mostly for seagrass beds. Instead of increasing like oyster beds, seagrass areas tend to decrease in time. Based on aerial photography past situations have been successfully reconstructed for seagrass beds in Australia (Larkum & West 1990, Kendrick et al. 2002) and the USA (Lathrop et al. 2001). From their reconstructions they could conclude an overall decrease in the area of



Figure 4. Oyster map 2002.



Figure 5. Intertidal oyster bed in the Eastern Scheldt.

seagrass beds. An increase of Pacific oyster area can be concluded from the results of this study. Figure 5 shows an example of a large intertidal oyster bed in the Eastern Scheldt.

Field survey is an expensive but necessary aspect of remote sensing. Field data are needed to describe habitats present in an area, identify locations of habitats in the imagery, and to provide an independent evaluation of the thematic accuracy of output maps (Congalton 1991).

Remote sensing products can play an important role in the planning of field studies, particularly when charts or maps are poor (Green et al. 1996). A field study is used to verify results obtained with our aerial photographs.

Frequently, remote sensing studies concerning the inventory and mapping of tropical coastal resources do not include an assessment of accuracy. Of the technical papers reviewed by Green et al. (1996) only 25% presented a quantitative evaluation of the accuracy of the image classification. The accuracy or reliability found in this study (87%) is comparable to the accuracy found by Kristiansen (1997). He estimated the number of occupied Greylag Geese nests using aerial photographs, and visited the nesting site afterwards. Reliability of nest identification from images was found to be 93% correct. Mumby et al. (1997) found user's accuracies for aerial photographs of 76% for coral reefs, 58% for algae, 73% for sand, and 63% for seagrass. In this study oyster beds had a user's accuracy of 74%. Jaubert et al. (1999) used aerial photographs to determine alterations in dimensions of *Posidonia* (seagrass) beds. They used several wavebands to classify several habitats, and concluded that the probability of confusing one subject category with another was extremely small. They did not further quantify the reliability of their work.

This study shows that aerial photography can be an aid in surveying intertidal Pacific oyster beds. Reconstruction of the present situation shows that the chances of false positives (i.e., a spot located as an oyster bed without being an oyster bed, or surface areas being overestimated) are small. On the other hand, in some case more than half of the surface of oyster beds was missed

(false negatives). This can be caused by the fact that the photographs were taken in 1999 and 2000, and the field survey was performed in 2002. A second problem is the tide on the moment photographs were taken. If the tide is not low enough, then several beds can be missed (e.g., Slikken van Viane). This second problem could be tackled by comparing the extent of the exposed intertidal region in the photos with the maps of the beds from 2002. Unfortunately there are no useful reference points on most of the photographs which allow determination whether the tidal elevation at the time the photos were taken obscure any of the beds.

The reconstruction of the present situation shows an underestimation of the surface area of oyster beds, not only due to a time gap between photo and field survey. This means that the chance of an overestimation of the 1980 and 1990 situations are small, but there is a chance that the surface areas for 1980 and 1990 are underestimated. The different accuracies are comparable with accuracies found in other studies.

The technique presented here only covers the inventory of intertidal oyster beds. It's clear there will also be a large surface area of subtidal oyster beds, influencing the intertidal beds in several ways. There are, however, no estimates of these surface areas. It would be very useful to know the extent of the subtidal oyster beds, but it is of course impossible to use aerial photographs. To survey subtidal oysters, techniques like side scan sonar could be useful, but it is beyond the scope of this study to explore the possibilities.

In the maps shown the oyster beds are filled polygons, suggesting areas fully covered with oysters. In the field situation however, not all oysters in the beds are alive. But although dead oyster will not compete for food with other shellfish, they still occupy space and prevent settling of cockles and oysters (Smaal et al. 2001). For biomass estimation, aerial photographs cannot be used and field survey is still necessary.

In conclusion, digital photographs can be helpful in (re)constructing intertidal oyster bed maps. The years of 1980, 1990, and 2002 were mapped, and the development of intertidal oyster beds in the Eastern Scheldt can be shown. To make useful aerial photographs for the determination of surface areas of oyster bed, low tide and good clear weather are essential. In some cases the aerial photographs were malfunctioned after scanning or rotating. It is recommended to use digital, georeferated photographs in future.

This method has the potential to be helpful in estimating the size of surface areas of intertidal Pacific oyster beds. It could reduce the need for relatively intensive field surveys.

ACKNOWLEDGMENTS

The authors thank Jack Perdon and the crew of the Schollevaar for assistance in the field, Joris Geurts van Kessel and Annemieke van der Pluim (RIKZ) for making the aerial photographs of the Eastern Scheldt disposable, Pauline Kamermans and Karin Troost for improving the manuscript by critical comments, and John McDarby for improving the English.

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GROWTH AND SURVIVORSHIP OF NON-NATIVE (*CRASSOSTREA GIGAS* AND *CRASSOSTREA ARIAKENSIS*) VERSUS NATIVE EASTERN OYSTERS (*CRASSOSTREA VIRGINICA*)

JONATHAN H. GRABOWSKI,* CHARLES H. PETERSON, SEAN P. POWERS,
DAVID GASKILL AND HENRY C. SUMMERSON

University of North Carolina at Chapel Hill, Institute of Marine Sciences,
Morehead City, North Carolina 28557

ABSTRACT The decline of wild populations of the eastern oyster *Crassostrea virginica* from fishing impacts and disease combined with limited success in its culture has stimulated discussion among coastal managers about the risks and benefits of introducing non-native oysters in Maryland, Virginia, and North Carolina. Field experiments in 1999 to 2000 and 2001 to 2002 comparing growth, survivorship, and prevalence of disease in 2 non-native oysters, *C. gigas* and *C. ariakensis*, versus *C. virginica* in North Carolina estuaries demonstrated that in high-salinity (>25‰) waters, performance of *C. gigas* in culture greatly surpassed that of both of the other oysters (with growth 162.4% higher than *C. virginica* and 54.1% higher than *C. ariakensis* and survivorship 33.1% higher than *C. virginica* and 22.3% higher than *C. ariakensis*). *C. ariakensis* survivorship at these high salinity sites was highly variable and unpredictable even when using environmental covariates, and at salinities below ~10‰ this species did not grow, rendering its culture nonviable at low salinity. However, in waters of intermediate salinity (15‰ to 25‰), *C. ariakensis* outgrew both of the other 2 oysters (35.9% higher than *C. gigas* and 24.5% higher than *C. virginica*) and exhibited 42.1% higher survivorship than *C. gigas*. Although survivorship of *C. virginica* and *C. ariakensis* did not differ significantly at intermediate salinities, only *C. virginica* failed to achieve legally harvestable sizes and, based on its increasingly high susceptibility to death from disease with age, is likely to have experienced much greater mortality by the time of complete grow-out. Experimental elevation above the bottom augmented growth and survivorship of *C. ariakensis* most strongly, whereas *C. gigas* was not influenced by rack height. Before large-scale introduction of any non-native oyster occurs, the quantitative biologic results should first be incorporated into economic evaluations that weigh expected profitability and ecosystem benefits against the potential ecologic risks of introduction (both for wild release and for aquaculture of triploids).

KEY WORDS: *Crassostrea ariakensis*, *Crassostrea gigas*, *Crassostrea virginica*, economic feasibility, triploid, oyster disease, oyster growth, oyster survivorship

INTRODUCTION

Previous introductions of non-native species have often had severe consequences for ecologic communities, including reduction of the diversity, abundance and distribution of native fauna and flora (Carlton 1992, Ruiz et al. 2000). Consequently, prior to intentionally introducing any exotic fisheries species, managers should carefully weigh potential negative against positive ecological effects and evaluate under what conditions (aquaculture of nonreproductives versus wild release), if any, introduction may be economically and ecologically justifiable. A key component of this evaluation process is defining the rationale or need for introduction (Carriker 1992). Specifically, there must be clearly identified and scientifically defensible reasons why the native species is inadequate (Courtney & Robins 1989) and the introduced species is expected to have a high potential for success (Mann 1979, Rosenfield & Kern 1979, Mann et al. 1991).

Frustration with the slow pace of restoration efforts targeting wild populations of the eastern oyster *Crassostrea virginica* (Gmelin 1791) coupled with high mortality rates associated with culture of this species over the past several decades have resulted in advocacy by the shellfish industry to introduce non-native oysters in Maryland, Virginia, and North Carolina (Mann et al. 1991, Byrne 1996, Shatkin et al. 1997). Two species, the Pacific oyster, *C. gigas* (Thunberg 1793), and the Suminoe oyster, *C. ariakensis* (Fujita 1913), have been proposed as candidates for triploid aquaculture and even wild introductions. Native to Japan and the Korean peninsula (Mann et al. 1991), *C. gigas* has been successfully introduced to France, Oregon, Washington, western Canada, Australia, and New Zealand (Shatkin et al. 1997) and currently ac-

counts for over 80% of the world's fishery production of oysters (Ayers 1991). Despite some taxonomic confusion with *C. rivularis*, the native distribution of *C. ariakensis* is believed to range from Pakistan through China to Japan, where it extends well into lower-salinity (i.e., <25‰) portions of estuaries (Breese & Malouf 1977, Langdon & Robinson 1996).

Resource managers currently face one of the most ecologically critical decisions in the history of environmental and fisheries management in the United States, whether *C. gigas* or *C. ariakensis* should be either intentionally released to propagate in the wild or cultured as nonreproductives in controlled aquaculture settings (National Research Council 2003). Some past studies provide information on and discussions of potential ecologic risks and perceived ecosystem (e.g., enhanced bio-filtration rates) and fisheries benefits of the 2 types of introduction (Mann 1979, Andrews 1980, Mann et al. 1991, Gaffney & Allen 1992, Lipton et al. 1992, Byrne 1996, Gottlieb & Schweighofer 1996, Shatkin et al. 1997). Several scientists have emphasized that significant risks to local and regional ecosystems exist and have yet to be fully addressed. For instance, introduction of reproductively viable non-native oysters could lead to eventual invasion of other estuaries in neighboring states or regions of the United States. Because information on the biology of these 2 species is sparse, the NRC Committee on Non-native Oysters in the Chesapeake Bay recently recommended that further research be conducted on the performance of native versus non-native oyster species (National Research Council 2003).

Realization of the potential fisheries benefits of introducing non-native oysters depends on their biology within the estuaries of the eastern United States, their marketability (see Grabowski et al. 2003 for relevant comparative information on marketability), and the integrated bioeconomics. Previous studies performed in Ches-

*Corresponding author. E-mail: jgrabowski@gmri.org

peake Bay comparing the biology of *C. virginica* to either *C. ariakensis* or to *C. gigas* (Barber & Mann 1994, Calvo et al. 1999, Calvo et al. 2001) have documented higher resistance to *Perkinsus marinus* and *Haplosporidium nelsoni* and faster individual growth rates of both non-native species compared with *C. virginica* (Langdon & Robinson 1996, Calvo et al. 1999, Calvo et al. 2001), although the growth advantage seems to vary with salinity for *C. gigas*. However, direct comparison of the two non-native oyster species has yet to be conducted within the eastern United States and neither non-native species has been experimentally cultured in North Carolina. Here we present results of field trials covering the full range of potentially viable salinity regimes in coastal North Carolina. These trials were designed to assess the growth, survivorship, and disease prevalence and severity of the two non-native species under consideration for introduction and contrast these results to those obtained simultaneously for *C. virginica*. In addition, we tested if elevating oysters off the bottom differentially affects survival and growth of native versus non-native oysters.

MATERIALS AND METHODS

A series of experiments was conducted to compare growth, survivorship, and disease (dermo, *P. marinus*) prevalence and severity among native *C. virginica* (eastern oyster) and 2 non-native species, *C. gigas* (Pacific oyster) and *C. ariakensis* (Suminoe oyster), in North Carolina from 1999 to 2002. For each experiment, triploid seed *C. gigas* and *C. ariakensis* were obtained from the quarantine hatchery at the Virginia Institute of Marine Sciences (VIMS) and compared with diploid *C. virginica* obtained from Leslie Lee, Sloop Point Seafood, Hampstead, North Carolina. Triploid non-native oysters were raised at VIMS until achieving approximately 2–6 cm shell height (SH) in size, and tested for ploidy and disease status. Disease-free triploid oysters were shipped to the University of North Carolina-Institute of Marine Sciences (UNC-IMS), where they were held in upwellers prior to initiating field trials. Native oysters were raised at Sloop Point Seafood in raceways until the inception of each experimental trial. Oysters were then cultured in 4.8-mm mesh polyethylene bags that were elevated above the bottom using racks constructed from iron bars and located at approximately 0.1–0.5 m below mean low water (MLW) in research sanctuaries throughout coastal North Carolina.

First Series of Experiments (1999–2000)

Grow-out experiments were performed in 1999 to 2000 to compare growth and survivorship of *C. virginica* versus *C. ariakensis* and *C. virginica* versus *C. gigas* in separate experiments. In December 1999, *C. ariakensis* (mean SH \pm 1 SE = 54.8 \pm 0.9 mm) and *C. virginica* (42.6 \pm 1.0 mm) were placed in 4.8-mm polyethylene (43 \times 48 \times 4 cm) bags on 15 cm-high racks at 1 high-salinity site (>25‰; Chadwick Bay) and 1 site with low (<10‰; Broad Creek) salinity (Fig. 1, Table 1). Abnormal environmental conditions following Hurricane Floyd resulted in extremely low salinity levels at Broad Creek in 2000 (Peterson 2000). Three bags of each species were deployed at each site with 52 oysters per bag. Living oysters were subsequently measured (SH) and counted to assess size and survivorship in March, June, and September/October 2000 at both sites.

C. gigas (31.4 \pm 0.8 mm) and *C. virginica* (29.5 \pm 0.6 mm) were placed in 6 polyethylene bags (50 oysters per bag, 3 bags per species) and deployed in February 2000 on 15 cm-high racks at

each of 2 high-salinity sites in Waters Bay and Chadwick Bay (see Fig. 1). Deployment time for *C. gigas* differed from that of *C. ariakensis* described earlier because of availability of hatchery seed. *C. gigas* was not planted at Broad Creek because its poor performance at low salinities is already well documented (Calvo et al. 1999). Living oysters for the *C. virginica*/*C. gigas* contrast were subsequently measured (SH) and counted in May and August 2000 at both sites. For both the *C. virginica*/*C. ariakensis* and *C. virginica*/*C. gigas* experiments, oyster bags were washed with pressurized water and scrubbed with wire brushes to remove accumulated mud and fouling organisms during each sampling visit.

Second Series of Experiments (2001–2002)

The second series of experiments was initiated in April 2001 and included comparisons of growth, survivorship, *P. marinus* infection and *Polydora* spp. infestation among *C. ariakensis*, *C. gigas*, and *C. virginica*. Three bags of 70 *C. ariakensis* (31.8 \pm 1.0 mm) and 3 bags of 70 *C. virginica* (20.6 \pm 0.3 mm) were placed at each of 4 high-salinity (>25‰; Topsail Sound, Waters Bay, Chadwick Bay and Newport River, see Fig. 1) and 3 intermediate-salinity (15‰ to 25‰; Bay River, Broad Creek and Swan Quarter) sites. In addition, 3 bags of 70 *C. gigas* (18.7 \pm 0.3 mm) were placed at each of the high- and 1 intermediate-salinity (Bay River) sites to determine if slightly reduced salinities negatively impact *C. gigas* growth and survivorship in North Carolina. Bags (4.8-mm mesh, 43 \times 48 \times 4 cm) containing each set of seed oysters were placed on 15 cm-high rebar racks 0.1–0.5 m below MLW. To determine if height above the bottom affects native or non-native oyster growth and survivorship, 3 bags of *C. virginica*, 3 bags of *C. gigas*, and 3 bags of *C. ariakensis* were planted on racks at each of 2 additional heights (on the seabed and 38 cm above the seabed) at 2 sites (Chadwick Bay and Newport River).

Living oysters at high salinities were subsequently measured (SH) and counted in June, October, and November 2001. Living oysters at intermediate salinities were measured (SH) in June and October 2001, and February 2002. Salinity (‰), dissolved oxygen (mg/L), and water temperature (°C) at 0–25 cm beneath the water surface were measured monthly at each site from May to August 2001 during the experiment. Bags were cleaned with pressurized water and brushes monthly during the summer and seasonally during the fall and winter.

At each site, up to 24 oysters (4–8 oysters from each bag per species) were tested in August and October 2001 for prevalence and intensity of the oyster disease *P. marinus* and shell infestation rates by the mud worm *Polydora* spp. When testing for *P. marinus* infections, a 3–5-mm-long section of the rectum was removed from each oyster and analyzed for the presence and intensity of *P. marinus* using Ray's fluid Thioglycollate medium (RTFM) assays (Ray 1952, Ray 1963, Paynter & Burrenson 1991). Infection intensity was calculated using the method described by Ray (1954) and Mackin (1962), with infection intensity categorized into the following groups: (0) absent, (1) light, (3) moderate, (5) heavy (Calvo et al. 1999, Lenihan et al. 1999). Average weighted intensity of *P. marinus* then was calculated for each species at each site by multiplying the number of oysters with each infection level by its infection intensity and dividing this sum by the total number of oysters tested. Intensity of *Polydora* spp. shell infestation was rated on a scale of 0 to 4 to describe the approximate percentage of the external oyster shell (right valve only) covered by mud worm tubes ([0] absent, [1] <25%, [2] 25% to 50%, [3] 50% to

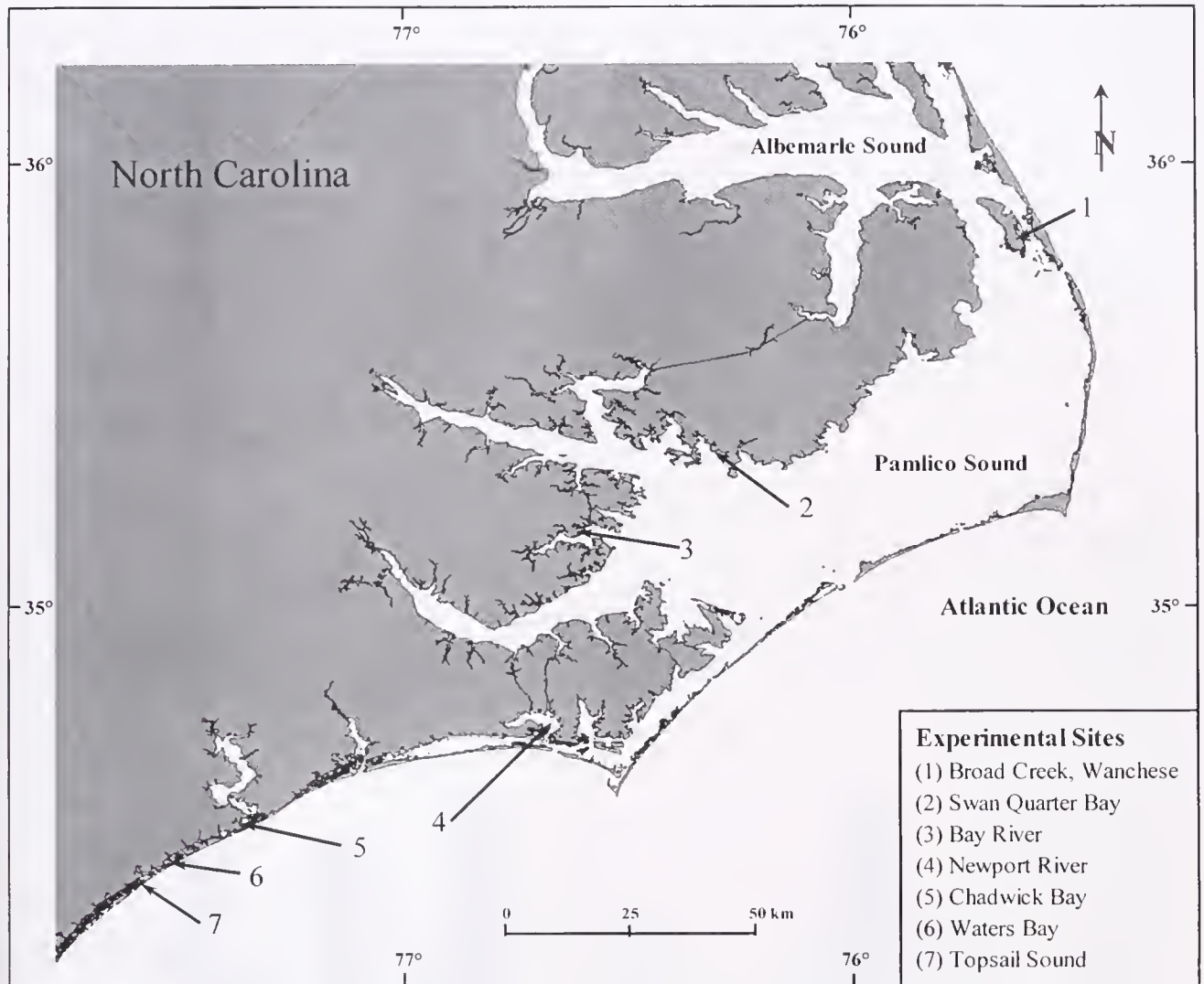


Figure 1. Location of experimental sites where native and non-native oysters were cultivated in 1999 to 2000 and 2001 to 2002 in coastal North Carolina, USA.

75%, and [4] >75% coverage). Weighted intensity was calculated by multiplying the number of oysters with each intensity level by their respective infestation intensity and dividing the sum by the total number of oysters tested.

Statistical Analyses

A series of multifactor ANOVAs was performed to test if growth and survivorship varied between or among oyster species

TABLE 1.

Mean and range of salinity, dissolved oxygen, and temperature from 25 cm below the water surface at field (± 0.1 – 0.5 m below MLW) sites in North Carolina during the second experiment. Each parameter was recorded monthly between May and August of 2001.

Site	County (NC)	Latitude	Longitude	Salinity (‰)		Dissolved Oxygen (mg/L)		Temperature (°C)	
				Mean	Range	Mean	Range	Mean	Range
Broad Creek, Wanchese	Dare	N35°50.530'	W75°37.170'	18	15–22	5.9	4.4–7.6	27.1	21.7–30.1
Swan Quarter Bay	Hyde	N35°23.217'	W76°19.618'	16	15–16	7.2	6.3–8.3	28.6	24.5–30.5
Bay River	Pamlico	N35°11.032'	W76°36.529'	18	16–20	5.5	4.0–7.6	26.7	20.9–29.7
Newport River	Carteret	N34°44.689'	W76°39.679'	34	31–36	5.4	4.0–6.3	25.9	21.0–28.2
Chadwick Bay	Onslow	N34°31.603'	W77°22.574'	38	35–40	5.5	4.3–8.3	25.6	19.5–29.2
Waters Bay	Onslow	N34°26.941'	W77°32.248'	38	35–40	6.6	4.4–9.5	24.0	19.6–27.8
Topsail Sound	Pender	N34°24.417'	W77°35.848'	38	35–40	5.8	3.7–7.7	26.8	19.3–30.8

and whether these patterns were modified by grow-out site. Cochran's test for homogeneity of variances was conducted on all main effects prior to each analysis (Underwood 1981). For datasets that violated this assumption at α of 0.05, fourth-root transformations were performed and transformed data were tested. For experiments conducted in 1999 and 2000, separate 2-way ANOVAs were performed on oyster growth (final SH minus initial SH) and survivorship with site (Chadwick Bay and Broad Creek) and species (*C. ariakensis* and *C. virginica*) as fixed factors. A second set of separate 2-way ANOVAs was performed on oyster growth and survivorship with site (Chadwick Bay and Waters Bay) and species (*C. gigas* and *C. virginica*) as fixed factors. For experiments initiated in 2001, we conducted a series of analyses to compare growth and survivorship of the 3 oyster species within each salinity regimen. At relatively high salinities, we analyzed the effects of site (Chadwick Bay, Newport River, Topsail Sound, and Waters Bay) and species (*C. ariakensis*, *C. gigas*, and *C. virginica*) on growth and survivorship using separate 2-way ANOVAs with fixed factors. The effect of species (*C. ariakensis*, *C. gigas*, and *C. virginica*) on oyster growth and survivorship at Bay River (intermediate salinity) was analyzed using separate 1-way (fixed factor) ANOVAs. For the other 2 intermediate-salinity sites, 2-way ANOVAs were conducted on growth and survivorship with site (Swan Quarter Bay and Broad Creek) and species (*C. ariakensis* and *C. virginica*) as fixed factors. To test the effect of elevating oysters on their growth and survivorship, a 3-way ANOVA was

performed with site (Chadwick Bay and Newport River), species (*C. ariakensis*, *C. gigas*, and *C. virginica*), and elevation (bottom, low, and high) as fixed factors. Student-Newman-Keuls (SNK) *post hoc* tests at α of 0.05 were conducted on all main effects. If an interaction proved significant in 2-way ANOVAs, SNK tests were performed among treatments within each level of a factor. The SNK test was selected because we conducted a balanced experiment with *a priori* predictions and fixed factors (Day & Quinn 1989).

RESULTS

First Series of Experiments

Results of the contrasts between *C. ariakensis* and *C. virginica* varied between the (low-salinity) Broad Creek and (high-salinity) Chadwick's Bay sites. *C. ariakensis* deployed in December grew by September from 55.3 mm SH to 56.9 ± 0.6 mm (mean \pm 1 standard error) at Broad Creek and from 54.3 to 99.3 ± 1.9 mm at Chadwick Bay, while *C. virginica* increased during this period from 42.5 to 51.0 ± 2.2 mm at Broad Creek and from 42.6 to 71.0 ± 0.9 mm at Chadwick Bay. ANOVA revealed a significant effect of the interaction between site and species ($F_{1,8} = 79.1$, $P < 0.0001$; Fig. 2a) on oyster growth (i.e., change in shell height). *C. ariakensis* grew 16.6 mm more than *C. virginica* at the high-salinity Chadwick Bay, but *C. virginica* outgrew *C. ariakensis* by 6.8 mm at the low-salinity Broad Creek (SNK *post hoc* compari-

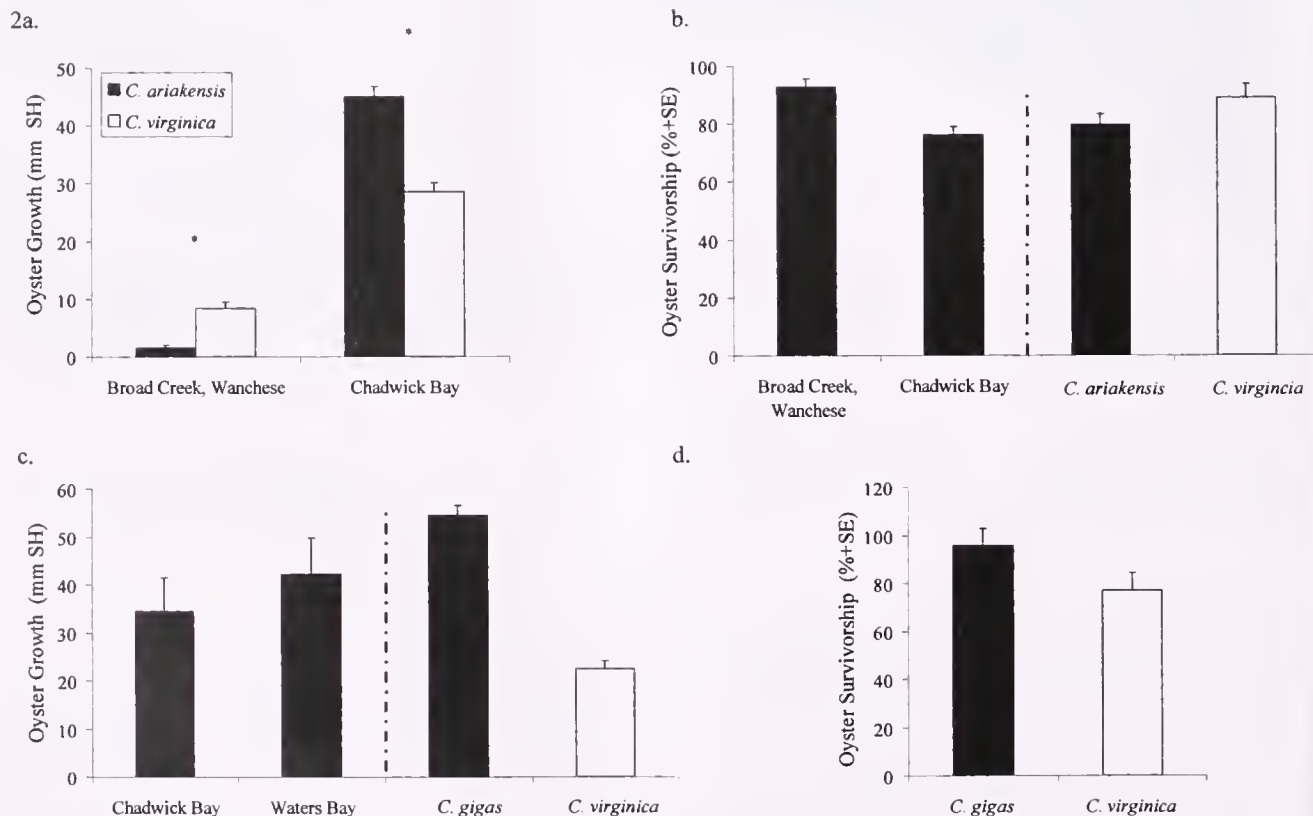


Figure 2. (a) *C. ariakensis* and *C. virginica* growth (final SH to initial SH) from December 1999 to September 2000 at Broad Creek and Chadwick Bay sites (+SE; $n = 3$). Significance levels (* $P < 0.05$; ns $P > 0.05$) presented above bars are from SNK *post hoc* tests conducted to explore the cause of the significant species \times site interaction. (b) Oyster (both *C. ariakensis* and *C. virginica*) survivorship at each of the 2 sites and survivorship of *C. ariakensis* versus *C. virginica* after 9 mo of culture (+SE; $n = 6$). (c) Oyster (both *C. gigas* and *C. virginica*) growth at Chadwick Bay versus Waters Bay and *C. gigas* versus *C. virginica* growth from February to August in 2000 (+SE; $n = 6$). (d) *C. gigas* versus *C. virginica* survivorship after 7 mo of oyster culture at Chadwick Bay and Waters Bay (+SE; $n = 6$).

sons; see Fig. 2a). ANOVA revealed that the interaction between site and species did not affect oyster survivorship ($F_{1,8} = 0.3$, $P = 0.58$). Oyster survivorship at Chadwick Bay was 16.5% higher than at Broad Creek (site effect: $F_{1,8} = 28.4$, $P = 0.0007$), and survivorship of *C. virginica* was 9.4% higher than that of *C. ariakensis* (species effect: $F_{1,8} = 9.1$, $P = 0.02$; see Fig. 2b).

Results of the contrasts between *C. gigas* and *C. virginica* did not vary between sites, both of which had similarly high salinities. By the end of August 2000, *C. gigas* deployed in January 2000 grew from 31.3 to 81.3 ± 1.1 mm SH at Chadwick Bay and from 31.5 to 90.2 ± 2.1 mm at Waters Bay, whereas *C. virginica* grew from 29.8 to 48.8 ± 1.7 mm at Chadwick Bay and from 29.1 to 54.8 ± 1.7 mm at Waters Bay. For the contrast between *C. gigas* and *C. virginica*, ANOVA revealed no significant effect of the interaction between site and species on either growth ($F_{1,8} = 0.4$, $P = 0.56$) or survivorship ($F_{1,8} = 0.4$, $P = 0.53$). *C. gigas* grew more than *C. virginica* at both sites (species effect: $F_{1,8} = 455.3$, $P < 0.0001$), and oyster growth was higher at Waters Bay for both species (site effect: $F_{1,8} = 26.6$, $P = 0.0009$; see Fig. 2c). Survivorship of *C. gigas* was 18.7% higher than that of *C. virginica* across both sites (species effect: $F_{1,8} = 8.8$, $P = 0.02$; see Fig. 2d). Thus, in these high-salinity sites *C. gigas* grew faster and survived better than the native oyster.

Second Series of Experiments

Salinity, Temperature, and Dissolved Oxygen

Physical parameters were quantified in the summer of 2001 to indicate how variation in these factors might influence patterns of

oyster growth and mortality. Between May and August 2001, mean salinity was 38‰ at Chadwick Bay, Waters Bay, and Topsail Sound and 34‰ at Newport River sites (Table 1). Mean dissolved oxygen ranged from 5.4 to 6.6 mg/L and mean water temperature from 24.0°C to 26.8°C during this period at the high-salinity sites (Table 1). Between May and August 2001, mean salinity was 18‰, dissolved oxygen 5.5 mg/L, and water temperature 26.7°C at Bay River (Table 1). Mean salinity at Swan Quarter Bay (16‰) was slightly lower than at Broad Creek (18‰) between May and August 2001 (Table 1). Mean dissolved oxygen was higher than all other sites at Swan Quarter Bay (7.2 mg/L), and this was the only site for the entire study where relatively low (<4.5 mg/L) dissolved oxygen levels were never recorded (Table 1). Finally, mean water temperature was slightly higher at Swan Quarter Bay (28.6°C) than at Broad Creek (27.1°C; Table 1).

High-salinity Sites

C. ariakensis versus *C. gigas* versus *C. virginica*. Growth and survivorship differed among non-native and native oysters in this set of trials. From April to November 2001, *C. gigas* in high salinity grew from 19.2 to 101.4 ± 2.3 mm SH (means of all 4 sites), *C. ariakensis* from 31.6 to 86.0 ± 2.2 mm, and *C. virginica* from 20.7 to 52.4 ± 1.9 mm. The interaction between site and species did not affect oyster growth ($F_{6,24} = 2.0$, $P = 0.11$), but each main effect was significant (site: $F_{3,6} = 25.8$, $P < 0.0001$; species: $F_{2,6} = 346.3$, $P < 0.0001$). Oyster growth at Waters Bay was greater than all other sites, which did not differ (SNK *post hoc* comparisons; Fig. 3a). *C. gigas* growth was greater

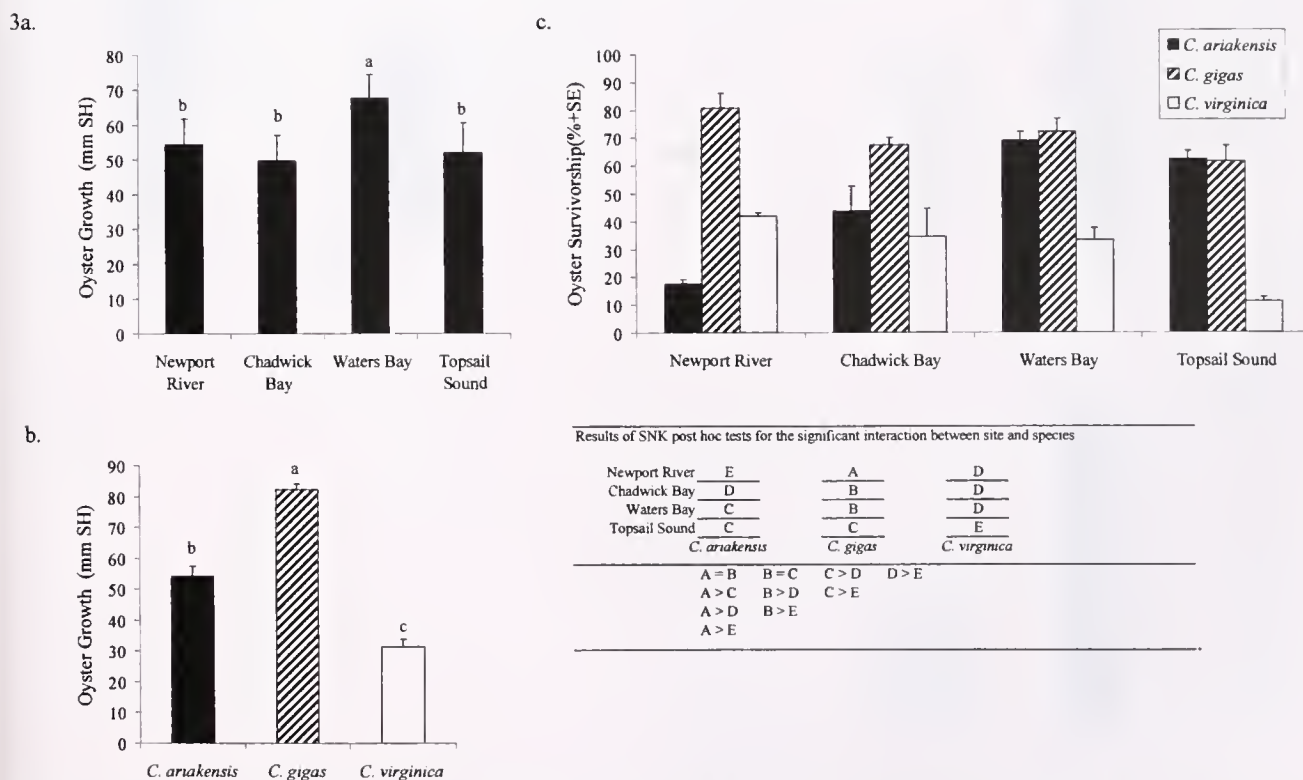


Figure 3. Culture of all 3 species at sites with high salinities from April to November in 2001. (a) The effect of site on oyster growth (final SH to initial SH) of all 3 species combined after 7 mo of culture (+SE; $n = 9$). Sites with different letters above error bars are significantly different at $P < 0.05$ (SNK *post hoc* tests). (b) The effect of species on growth of each species during oyster culture (+SE; $n = 12$). (c) The interaction between site and species on oyster survivorship at high salinities (+SE; $n = 3$).

than that of the other 2 species, and *C. ariakensis* outgrew *C. virginica* (see Fig. 3b). ANOVA revealed a significant interaction of site and species for oyster survivorship ($F_{6,24} = 12.8$, $P < 0.0001$; see Fig. 3c). Survivorship of *C. gigas* exceeded that of *C. ariakensis* at the Chadwick Bay and Newport River sites, and was greater than that of *C. virginica* at all 4 sites (Fig. 3c). Survivorship of *C. ariakensis* exceeded that of *C. virginica* at Topsail Sound and Waters Bay, but was less than that of *C. virginica* at Newport River (Fig. 3c). Finally, survivorship of *C. ariakensis* and *C. virginica* did not differ at Chadwick Bay (Fig. 3c).

Intermediate-salinity Sites

Bay River (*C. ariakensis* versus *C. gigas* versus *C. virginica*). In contrast to the results at the high-salinity sites, both *C. ariakensis* and *C. virginica* exhibited higher growth and survivorship than *C. gigas* at Bay River. From April 2001 to February 2002, *C. ariakensis* had grown from 32.5 to 82.9 ± 1.7 mm SH. *C. virginica* from 20.1 to 56.3 ± 0.02 mm, and *C. gigas* from 16.5 to 43.2 ± 1.1 mm. *C. ariakensis* outgrew both of the other species, and growth of *C. virginica* was greater than that of *C. gigas* (SNK *post hoc*

comparisons; $F_{2,6} = 88.0$, $P < 0.0001$; Fig. 4a). Survivorship did not differ between *C. ariakensis* and *C. virginica*, but survivorship of each was greater than that of *C. gigas* ($F_{2,6} = 52.4$, $P = 0.0002$; see Fig. 4b).

Broad Creek and Swan Quarter Bay (*C. ariakensis* versus *C. virginica*). The pattern exhibited at the other intermediate-salinity site (Bay River) of higher growth of *C. ariakensis* than *C. virginica* but equivalent survivorship was replicated in this set of trials. From April 2001 to February 2002, *C. ariakensis* grew from 31.4 to 62.1 ± 1.0 mm SH at Broad Creek and from 31.1 to 80.7 ± 3.5 mm at Swan Quarter Bay, whereas *C. virginica* increased from 18.8 to 48.8 ± 1.0 at Broad Creek and from 18.9 to 56.5 ± 0.6 mm, respectively, at the 2 sites. ANOVA revealed a significant effect of the interaction between site and species for oyster growth ($F_{1,8} = 7.5$, $P = 0.03$; Fig. 4c). *C. ariakensis* outgrew *C. virginica* at Swan Quarter Bay, but not at Broad Creek (SNK *post hoc* comparisons; Fig. 4c). For both species, growth was greater at Swan Quarter Bay than at Broad Creek (Fig. 4c). ANOVA revealed no significant effect on oyster survivorship of either main effect (site: $F_{1,8} = 0.5$, $P = 0.51$; species: $F_{1,8} = 1.4$, $P = 0.27$) or the interaction between site and species ($F_{1,8} = 2.8$, $P = 0.13$). Mean survivor-

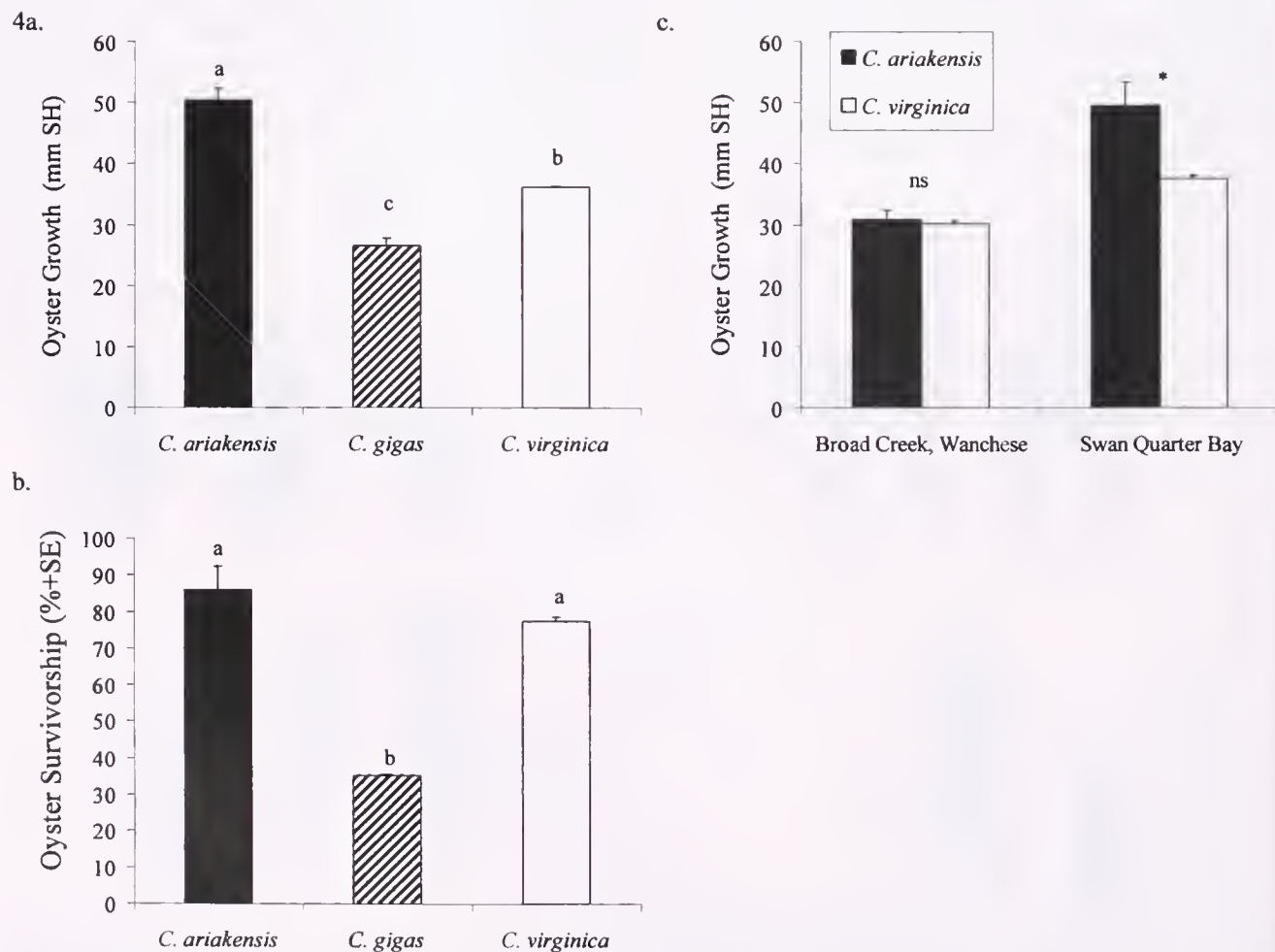


Figure 4. Oyster culture at intermediate-salinity sites from April 2001 to February 2002. (a) Growth and (b) Survivorship of all 3 species at Bay River from April 2001 to February 2002 (+SE; $n = 3$ for both graphs). Species with different letters above error bars are significantly different at $P < 0.05$. (c) *C. ariakensis* and *C. virginica* growth at Broad Creek and Swan Quarter Bay from April 2001 to February 2002 (+SE; $n = 3$). Significance levels (* $P < 0.05$; ns $P > 0.05$) presented above bars are from SNK *post hoc* tests conducted to explore the cause of the significant site X species interaction.

ship at these 2 sites was 74.1 ± 4.2 for *C. ariakensis* and 83.4 ± 7.3 for *C. virginica*.

Oyster Elevation Experiment

C. ariakensis versus *C. gigas* versus *C. virginica*. Elevating oysters enhanced the growth of *C. ariakensis* more than that of the other two oyster species. ANOVA revealed a significant effect of the 3-way interaction among site, species, and elevation for oyster growth (Table 2 and Fig. 5a). Height of oysters did not affect *C. gigas* growth at either site (SNK *post hoc* comparisons: Table 2,

Table 3 and Fig. 5a). *C. ariakensis* growth was greater on high racks than on the bottom at both sites, and was also greater on high-rack racks than on low racks at Chadwick Bay (Table 2, Table 3, and Fig. 5a). *C. virginica* growth was greatest on high racks, intermediate on low racks, and lowest on the bottom at Newport River, but did not differ at Chadwick Bay (Table 2, see Fig. 5a). *C. gigas* growth was greater than that of the other 2 species for all 3 elevations at both sites (Table 2, Table 3, and Fig. 5a). *C. ariakensis* growth was greater than that of *C. virginica* at all 3 elevations at both sites except for the bottom at Chadwick Bay, where the 2 species did not differ (see Table 2 and Fig. 5a).

TABLE 2.

The effect of site (Newport River and Chadwick Bay), species (*C. ariakensis*, *C. gigas*, and *C. virginica*), and elevation (bottom, low rack, and high rack) on oyster growth (change in shell height: SH) and survivorship in 2001 analyzed using separate 3-way ANOVAs.

	Oyster Growth (SH)					Oyster Survivorship			
	df	SS	F	P		df	SS	F	P
Site	1	0.001	3.6	0.06	1	0.079	7.4	0.01	
Species	2	0.302	522.4	<.0001	2	1.671	78.2	<.0001	
Height	2	0.009	15.9	<.0001	2	0.340	15.9	<.0001	
Site \times species	2	0.002	3.8	0.03	2	0.333	15.6	<.0001	
Site \times height	2	0.001	2.0	0.16	2	0.069	3.2	0.05	
Species \times height	4	0.010	8.5	<.0001	4	0.132	3.1	0.03	
Site \times species \times height	4	0.005	4.6	0.004	4	0.019	0.4	0.78	
Residual	36	0.010			36	0.385			

3-way interaction for oyster growth: site \times species \times height

	D	C	B	D	D	B
<i>C. ariakensis</i>	D	C	B	D	D	B
<i>C. gigas</i>	A	A	A	A	A	A
<i>C. virginica</i>	I	H	F	E	G	F
	Bottom	Low	High	Bottom	Low	High
Newport River						
A > B	B = C	C = D	D = E	E = F	F = G	G = H
A > C	B > D	C = E	D > F	E = G	F > H	G > I
A > D	B > E	C > F	D > G	E > H	F > I	
A > E	B > F	C > G	D > H	E > I		
A > F	B > G	C > H	D > I			
A > G	B > H	C > I				
A > H	B > I					
A > I						
Chadwick Bay						

2-way interaction for oyster survivorship: site \times species

Newport River	C	A	C
Chadwick Bay	B	A	C
	<i>C. ariakensis</i>	<i>C. gigas</i>	<i>C. virginica</i>
A > B	B = C		
A > C			

2-way interaction for oyster survivorship: species \times height

<i>C. ariakensis</i>	E	E	B
<i>C. gigas</i>	A	A	A
<i>C. virginica</i>	E	D	C
	Bottom	Low	High
A > B	B > C	C = D	D = E
A > C	B > D	C > E	
A > D	B > E		
A > E			

Notes: Provided also are results of SNK *post hoc* tests (using Bonferroni's adjustment for multiple contrasts to maintain experiment-wise $\alpha = 0.05$) for each significant interaction at $P < 0.05$ for the above analyses.

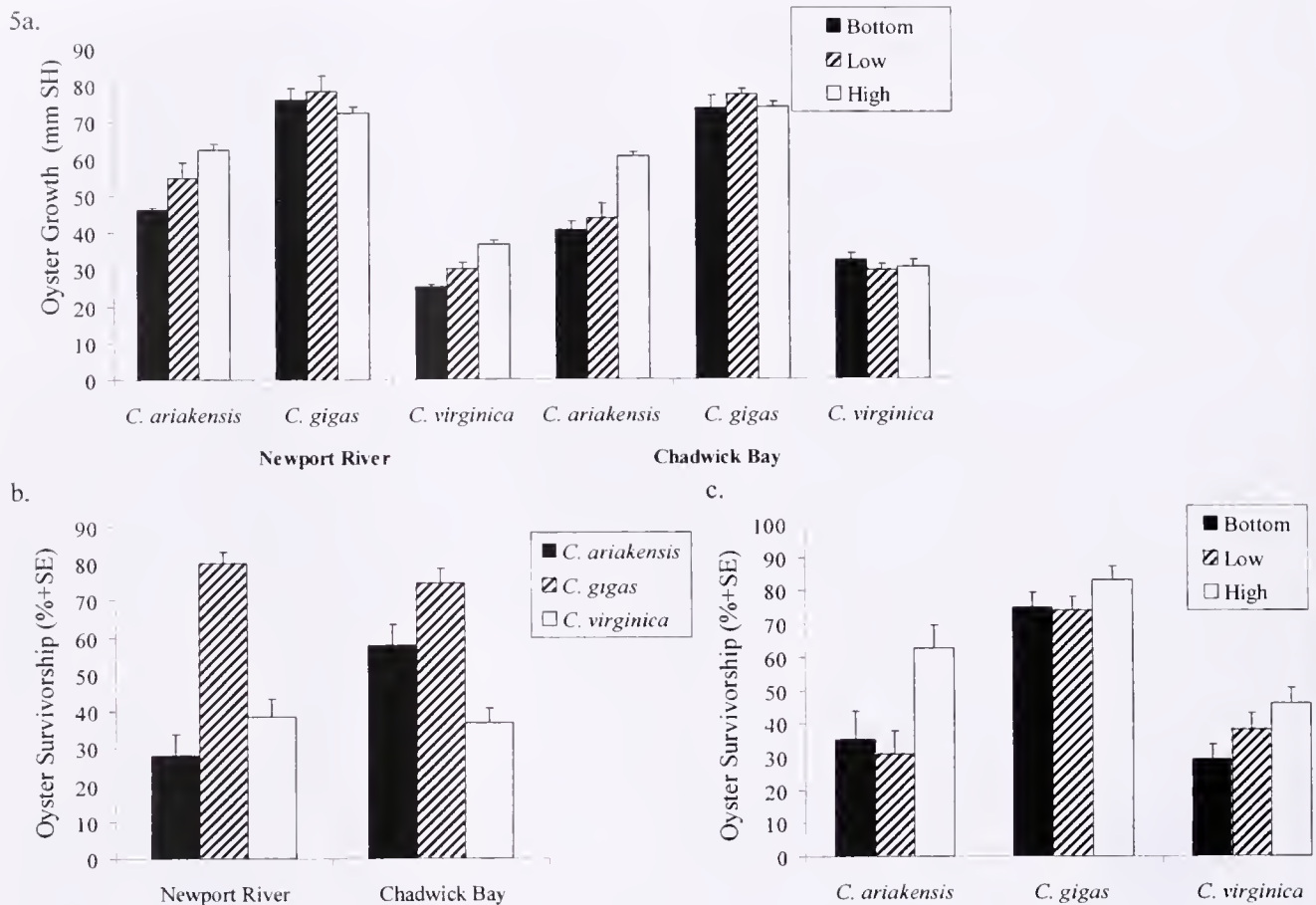


Figure 5. Culture of all 3 species on racks of differing elevations and on the bottom from April to November of 2001 at Newport River and Chadwick Bay. (a) The three-way interaction among site, species and elevation on oyster growth (+SE; $n = 3$). (b) The two-way interaction between site and species on oyster survivorship (+SE; $n = 9$). (c) The two-way interaction between species and elevation on oyster survivorship (+SE; $n = 6$). See Table 2 for results of SNK *post hoc* tests for each of the 3 graphs.

Although ANOVA of oyster survivorship revealed no 3-way interaction among site, species and elevation, all 3 2-way interactions were significant (site X species, species X elevation) or marginally significant (site X elevation; Table 2 and Fig. 5b,c). For the interaction between site and species, *C. gigas* survivorship was significantly higher than that of the other 2 species at both sites (SNK *post hoc* comparisons; Table 2 and Fig. 5b). *C. ariakensis* survivorship was greater than that of *C. virginica* at Chadwick Bay, but survivorship of the 2 species did not differ at Newport

River (Table 2 and Fig. 5b). For the interaction between species and elevation, elevating oysters did not affect survivorship of *C. gigas* (Table 2 and Fig. 5c). In contrast, elevating *C. ariakensis* from the bottom or low racks to high racks increased its survivorship (Table 2 and Fig. 5c). Survivorship of *C. virginica* was greater on high racks than on the bottom, but did not differ from low racks (Table 2 and Fig. 5c). *C. gigas* survivorship was greater than that of the other 2 species at all 3 elevations (Table 2 and Fig. 5c). Survivorship was greater for *C. ariakensis* than for *C. virginica*

TABLE 3.

Initial and final oyster sizes (shell height) of oysters grown at each elevation (bottom, low rack, and high rack) from April to November of 2001 at high-salinity sites (Newport River and Chadwick Bay) in North Carolina.

Site	Elevation	<i>C. ariakensis</i>		<i>C. gigas</i>		<i>C. virginica</i>	
		Initial SH	Final SH	Initial SH	Final SH	Initial SH	Final SH
Newport River	Bottom	30.4	76.8 (0.6)	20.9	97.2 (3.5)	20.4	45.7 (0.5)
	Low	28.9	83.9 (3.8)	19.6	98.0 (4.1)	20.7	50.8 (1.7)
	High	30.4	89.9 (1.1)	20.9	91.7 (1.6)	20.4	57.4 (1.3)
Chadwick Bay	Bottom	30.9	71.5 (1.9)	18.5	91.1 (3.3)	20.1	52.4 (2.0)
	Low	31.0	74.9 (2.8)	19.5	96.7 (1.3)	21.4	51.0 (2.2)
	High	30.9	89.4 (1.1)	18.5	93.4 (1.4)	20.1	50.7 (1.7)

only when oysters were raised on high racks (Table 2 and Fig. 5c). For the marginally significant (Table 2, $P = 0.05$) interaction between site and elevation, elevating oysters affected oyster survivorship only at Newport River, where survivorship of oysters was significantly greater on high racks than on the bottom.

Incidence of Oyster Disease and *Polydora* spp. Infestation

The prevalence (% of oysters infected) and intensity of *P. marinus* infection was extremely low at all sites for oysters tested in both August (1.7% were infected) and October 2001 (3.3% were infected; Table 4). In August, *P. marinus* was detected at only 1 to 2 sites for each of the 3 species, and its average prevalence for any species at any site was never greater than 10.0% (Table 4). In October, *P. marinus* was detected in *C. ariakensis* at 4 of 7 sites, and was most prevalent (16.7%) among *C. ariakensis* on low racks in Newport River and most intense (0.67) among *C. ariakensis* at Topsail Sound (Table 4). Of the 3 species, *P. marinus* was least prevalent and least intense among *C. gigas* and was detected only among *C. gigas* on high racks at Chadwick Bay in October (Table 4). *P. marinus* was detected in *C. virginica* at 4 of 7 sites in October (see Table 4).

In August 2001, the prevalence (% of oysters infected) and intensity of *Polydora* spp. infestation were greatest among *C. ariakensis*, intermediate among *C. gigas*, and almost nonexistent for *C. virginica* (Table 5). Prevalence and intensity of mud worms on *C. ariakensis* shells were very high at Broad Creek and Swan Quarter Bay, intermediate at Chadwick Bay, and very low at the other 4 sites (Table 5). Mud worm tubes were present on over half of *C. gigas* oysters tested at Chadwick Bay, but were present at only 1 of the other 4 sites (6.7% at Waters Bay; see Table 5). Waters Bay was the only site where mud worm tubes were present on *C. virginica* oysters (Table 5). In October 2001, mud worm

prevalence and intensity were greatest among *C. ariakensis* and *C. gigas*, and almost nonexistent among *C. virginica*. Mud worms were present on *C. ariakensis* and *C. gigas* shells at all sites except Waters Bay and Topsail Sound, and were most prevalent and intense at Chadwick Bay (Table 5). For *C. virginica*, mud worms were detected only at Chadwick Bay on high racks. In October, both infestation prevalence and intensity on shells of *C. ariakensis* and *C. gigas* were slightly greater on low racks than on either the bottom or high racks.

DISCUSSION

Critical to any decision on the introduction of non-native species for aquaculture, fisheries, or restoration of ecosystem services once provided by native species is an assessment of the biology of the candidate species in their prospective new environment. The primary motivations for introducing 1 of the 2 non-native oysters to Chesapeake Bay are their presumed resistance to *P. marinus* and *H. nelsoni*, with consequent survival advantages over the native oyster, and their high individual growth rates (National Research Council 2003). Previous studies comparing non-native oysters to *C. virginica* have found that the 2 non-native species tend to grow and survive better than the native oyster (Barber & Mann 1994, Calvo et al. 1999, Calvo et al. 2001), although reducing salinity can decrease or eliminate the growth and survivorship advantages of *C. gigas*. Barber and Mann (1994) demonstrated that growth and survivorship of *C. gigas* grown in the Chesapeake Bay were negatively impacted by salinities below 20‰. Similarly, Calvo et al. (1999) found that growth and survivorship of *C. gigas* in Chesapeake Bay were reduced at an intermediate salinity range of 15‰ to 25‰ in contrast to sites with salinities consistently above 25‰. They also noted that the individual growth rate of *C. gigas* was no longer greater than that of *C. virginica* at this intermediate-salinity

TABLE 4.

Prevalence and intensity of the oyster disease *Perkinsus marinus* among native and non-native oysters grown at 7 field sites in North Carolina. *P. marinus* prevalence and intensity were examined in August and October of 2001.

Experimental Height	Broad Creek	Swan Quarter Bay	Bay River	Newport River			Chadwick Bay			Waters Bay	Topsail Sound
	Low	Low	Low	Bottom	Low	High	Bottom	Low	High	Low	Low
August 2001											
<i>C. ariakensis</i>											
% infected	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	6.7%	0.0%	6.7%	0.0%
Weighted intensity ^a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.07	0.00
<i>C. gigas</i>											
% infected			5.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Weighted intensity			0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>C. virginica</i>											
% infected	0.0%	0.0%	0.0%	0.0%	10.0%	0.0%	0.0%	0.0%	0.0%	7.1%	0.0%
Weighted intensity	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.07	0.00
October 2001											
<i>C. ariakensis</i>											
% infected	7.1%	0.0%	0.0%	0.0%	16.7%	0.0%	0.0%	0.0%	14.3%	0.0%	13.3%
Weighted intensity	0.07	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.29	0.00	0.67
<i>C. gigas</i>											
% infected			0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	7.7%	0.0%	0.0%
Weighted intensity			0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.00
<i>C. virginica</i>											
% infected	13.3%	0.0%	6.7%	0.0%	0.0%	0.0%	14.3%	0.0%	0.0%	7.7%	0.0%
Weighted intensity	0.13	0.00	0.07	0.00	0.00	0.00	0.14	0.00	0.00	0.38	0.00

^a Infection intensity was calculated using the method described by Ray (1954) and Mackin (1962), with infection intensity categorized into the following groups: (0) absent, (1) light, (3) moderate, (5) heavy (Calvo et al. 1999, Lenihan et al. 1999). Average weighted intensity of dermo then was calculated for each species at each site by multiplying the number of oysters with each infection level by its infection intensity and dividing this sum by the total number of oysters tested.

TABLE 5.

Prevalence and intensity of mud worm *Polydora* spp. infestation among native and non-native oysters grown at 7 field sites in North Carolina. Mud worm infestation levels were quantified in August and October of 2001.

Experimental Height	Broad Creek	Swan Quarter Bay	Bay River	Newport River			Chadwick Bay			Waters Bay	Topsail Sound
	Low	Low	Low	Bottom	Low	High	Bottom	Low	High	Low	Low
August 2001											
<i>C. ariakensis</i>											
% w/ <i>Polydora</i>	100.0%	86.7%	0.0%	0.0%	0.0%	0.0%	33.3%	26.7%	23.8%	0.0%	0.0%
Weighted intensity	3.00	1.40	0.00	0.00	0.00	0.00	0.89	0.33	0.48	0.00	0.00
<i>C. gigas</i>											
% w/ <i>Polydora</i>			0.0%	0.0%	0.0%	0.0%	66.7%	40.0%	66.7%	6.7%	0.0%
Weighted intensity ^a			0.00	0.00	0.00	0.00	1.27	0.80	1.27	0.07	0.00
<i>C. virginica</i>											
% w/ <i>Polydora</i>	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	7.1%	0.0%
Weighted intensity	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00
October 2001											
<i>C. ariakensis</i>											
% w/ <i>Polydora</i>	28.6%	46.2%	60.0%	0.0%	16.7%	28.6%	92.3%	100.0%	64.3%	0.0%	0.0%
Weighted intensity	0.93	0.92	1.53	0.00	0.17	0.29	1.31	3.00	1.79	0.00	0.00
<i>C. gigas</i>											
% w/ <i>Polydora</i>			50.0%	13.3%	53.8%	0.0%	66.7%	100.0%	69.2%	0.0%	0.0%
Weighted intensity			1.60	0.13	0.77	0.00	1.67	2.00	1.54	0.00	0.00
<i>C. virginica</i>											
% w/ <i>Polydora</i>	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	9.1%	0.0%	0.0%
Weighted intensity	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00

Intensity of *Polydora* spp. shell infestation was rated on a scale of 0 to 4 to approximate the percentage of the oyster shell covered by mud worm tubes ([0] absent, [1] <25%, [2] 25–50%, [3] 50–75%, and [4] >75% coverage; Calvo et al. 1999). Weighted intensity was calculated by multiplying the number of oysters with each intensity level by their respective infestation intensity and dividing the sum by the total number of oysters tested.

level. Calvo et al. (2001) found low mortality rates for *C. ariakensis* over a wide spectrum of salinities (<15‰, 15‰ to 25‰, >25‰), suggesting that the physiology of *C. ariakensis* is influenced less by salinity than that of *C. gigas*. High *C. virginica* mortality rates in 2 of these studies (Barber & Mann 1994, Calvo et al. 2001) were primarily caused by the parasitic protozoan *P. marinus*, which is one of the largest impediments to native oyster aquaculture and recovery of native oyster fisheries in the estuaries of Maryland and Virginia. Prior to our study, growth and survivorship of the two non-native species had yet to be compared along the Atlantic coast of the United States, though Robinson and Langdon (1993) found that *C. gigas* growth was greater than that of *C. ariakensis* at sites on the West coast.

Results of our study provide clear evidence that the 2 non-native oysters, *C. gigas* and *C. ariakensis*, differ dramatically from one another and from the native eastern oyster, *C. virginica* in critical biologic rates (Table 6). As anticipated from previous studies done in the Chesapeake Bay (Barber & Mann 1994, Calvo et al. 1999), we found that *C. gigas* grows faster and survives at higher rates in high-salinity waters (25‰ to 36‰) than in 15‰ to 25‰ salinities. At the high-salinity sites, *C. gigas* exhibited consistent and substantial growth and survival advantages over the other 2 species (i.e., 162.4% higher growth than *C. virginica* and 54.1% higher than *C. ariakensis* and 33.1% higher survivorship than *C. virginica* and 22.3% higher than *C. ariakensis*). Thus, at high salinity, performance of the non-native *C. gigas* greatly exceeds that of the native eastern oyster in both biologic traits critical to production, namely growth and survivorship. From previous research (Barber & Mann 1994) and our own more limited data, the survivorship advantage of *C. gigas* could be related to greater resistance to *P. marinus* infection. At the 1 site of intermediate salinity (15‰ to 25‰) where we deployed *C. gigas*, it was sig-

nificantly outperformed in both growth and survival by the native eastern oyster, *C. virginica*. Therefore, any enthusiasm for introduction of *C. gigas* to Chesapeake Bay or the Pamlico Sound must be tempered by the realization that in the vast majority of the waters of these estuaries salinities favor the native eastern oyster.

Our results from deploying *C. ariakensis* in the small-scale grow-out trials confirm some previous conclusions from analogous research in Chesapeake Bay (Calvo et al. 2001) while providing new insights as well from direct contrasts with *C. gigas* and from our tests of elevation impacts. We first demonstrated in cold-season trials that salinity levels below 10‰ virtually inhibited all net growth and caused high mortality of *C. ariakensis*, thereby serving to help define one environmental and thus geographic limit to its successful culture. A site with salinity consistently below 10‰ proved unsuitable to achieve net growth in winter and simultaneously induced high mortality. The native eastern oyster actually significantly outperformed *C. ariakensis* at this low-salinity site in both growth and survival. However, the native oyster would have required an additional 3 to 4 y to achieve market size based on observed growth rates of *C. virginica* from this site, rendering such environments poor candidates for its culture also.

At sites of intermediate salinity (15‰ to 25‰), *C. ariakensis* significantly outgrew *C. gigas* by 35.9% and *C. virginica* by 24.5%. At intermediate salinity, *C. ariakensis* survivorship was 42.1% higher than that of *C. gigas*, but it did not differ significantly from *C. virginica*. However, the absence of a survival advantage of *C. ariakensis* over *C. virginica* in our study is misleading. By the time our trials were terminated, *C. ariakensis* had already reached a legally harvestable size (76.2 mm SH in North Carolina), whereas the more slowly growing native oyster had not. Using observed growth rates of *C. virginica* in our study, approximately 2–10 additional months of culture would have been re-

TABLE 6.

Summary of results from experimental culture of native (*C. virginica*) versus non-native (*C. ariakensis* and *C. gigas*) during 1999–2000 and 2001–2002 in North Carolina.

	Year	Site	Species Compared	Elevation	Results	
					Individual Growth	Survivorship
1. High-salinity (>25‰) sites						
Elevation experiment	1999–2000	Chadwick Bay	<i>C. ariakensis</i> versus <i>C. virginica</i>	Low elevation	aria > virg	virg > aria ^a
	1999–2000	Chadwick Bay	<i>C. gigas</i> versus <i>C. virginica</i>	Low elevation	gigas > virg	giga > virg
	1999–2000	Waters Bay	<i>C. gigas</i> versus <i>C. virginica</i>	Low elevation	gigas > virg	gigas > virg
	2001–2002	Waters Bay	All 3 species	Low elevation	gigas > aria > virg	aria = gigas > virg
	2001–2002	Topsail Sound	All 3 species	Low elevation	gigas > aria > virg	aria = gigas > virg
	2001–2002	Newport River	All 3 species	Bottom	gigas > aria > virg	gigas > aria = virg
	2001–2002	Newport River	All 3 species	Low elevation	gigas > aria > virg	gigas > virg > aria
	2001–2002	Newport River	All 3 species	High elevation	gigas > aria > virg	gigas > aria > virg
	2001–2002	Chadwick Bay	All 3 species	Bottom	gigas > aria = virg	gigas > aria = virg
	2001–2002	Chadwick Bay	All 3 species	Low elevation	gigas > aria > virg	gigas > aria = virg
	2001–2002	Chadwick Bay	All 3 species	High elevation	gigas > aria > virg	gigas > aria > virg
	2001–2002	Newport River	<i>C. ariakensis</i>	All 3 elevations	high = low > bottom	high > low = bottom
	2001–2002	Newport River	<i>C. gigas</i>	All 3 elevations	bottom = low = high	bottom = low = high
	2001–2002	Newport River	<i>C. virginica</i>	All 3 elevations	high > low > bottom	high > bottom
	2001–2002	Chadwick Bay	<i>C. ariakensis</i>	All 3 elevations	high > low = bottom	high > low = bottom
	2001–2002	Chadwick Bay	<i>C. gigas</i>	All 3 elevations	bottom = low = high	bottom = low = high
2001–2002	Chadwick Bay	<i>C. virginica</i>	All 3 elevations	bottom = low = high	high > bottom	
2. Intermediate-salinity (15–25‰) sites						
	2001–2002	Broad Creek, Wanchese	<i>C. ariakensis</i> versus <i>C. virginica</i>	Low elevation	aria = virg	aria = virg
	2001–2002	Swan Quarter Bay	<i>C. ariakensis</i> versus <i>C. virginica</i>	Low elevation	aria > virg	aria = virg
	2001–2002	Bay River	All 3 species	Low elevation	aria > virg > gigas	aria = virg > gigas
3. Low-salinity (<10‰) site ^b						
	1999–2000	Broad Creek, Wanchese	<i>C. ariakensis</i> versus <i>C. virginica</i>	Low elevation	virg > aria	virg > aria

^a *C. virginica* had not reached marketable size by the end of the experiment. Therefore, the 2–9 months of additional estimated grow-out would be expected to lead to much more mortality from dermo and other sources.

^b Abnormal environmental conditions following Hurricane Floyd resulted in extremely low salinity levels at Broad Creek in 2000 (Peterson 2000).

quired to achieve market size. The several additional months of warm water exposure required to complete grow-out of *C. virginica* would almost certainly have elevated its mortality, perhaps even dramatically if *P. marinus* infection had increased as expected (Lenihan et al. 1999).

At sites of high salinity, growth of *C. ariakensis* consistently and significantly exceeded that of the native *C. virginica* in all 5 trials where this contrast was set up. However, *C. ariakensis* survivorship was highly variable and unpredictable even using the environmental information on actual salinity, temperature, and DO variation that we collected. Over the 5 trials comparing *C. ariakensis* to *C. virginica* at high salinity, *C. virginica* survived at a significantly higher rate in 2 cases, *C. ariakensis* survived better in 2 cases, and no significant difference was detected in the remaining contrast. The high variability in the survivorship results for these 2 species at high salinity differs from the consistent advantage of *C. ariakensis* previously demonstrated in the Chesapeake Bay study of Calvo et al. (2001).

Comparison of triploid non-native oysters with diploid *C. virginica* could partly explain why non-native oysters outgrew *C. virginica* because reduced gamete production in triploids generally results in enhanced somatic growth (Barber & Mann 1994). Allen and Downing (1986) and Davis (1989) documented that triploid *C.*

gigas outgrow diploid *C. gigas*, particularly during the reproductive season. However, in a previous study with triploid *C. gigas* and *C. virginica*, *C. gigas* growth was nearly double that of triploid native oysters at high salinities (Calvo et al. 1999), suggesting that growth results in our study are only slightly confounded by differences in ploidy status among species.

A second potential limitation of this study was that the size and condition of oysters differed among species at the beginning of each experiment. In particular, *C. ariakensis* were approximately 10 mm (SH) larger than either of the other two species at the beginning of the experiment. *C. ariakensis* were raised at VIMS until they were large enough to be tested for ploidy status prior to use in this study. Because the proportional growth in oyster biomass increases with each incremental gain in shell height, an incremental gain in shell height for a larger oyster represents greater growth in biomass than the amount of biomass growth from a similar gain in shell height of a smaller oyster. Therefore, in this study growth rates of *C. ariakensis* are likely underestimated relative to the other two species. In particular, differences in growth rates between *C. gigas* and *C. ariakensis* were likely overestimated at high salinity sites, and *C. ariakensis* growth advantages over *C. virginica* and *C. gigas* (at low salinities) were probably underestimated. Another important consideration is that these oyster spe-

cies differ in morphology, so that an incremental change in SH for each species does not necessarily represent a uniform change in oyster biomass. Comparison of oysters tissue weights of larger (80–110 mm SH) oysters that did not differ in SH determined that *C. ariakensis* tissue weight was approximately twice that of *C. virginica* and one-third greater than *C. gigas* tissue weight (Grabowski et al. 2003). Thus, comparing growth rates by quantifying changes in shell height also underestimated *C. ariakensis* growth relative to the other two species.

Our experiment that varied the elevation of oysters from culture on the bottom to racks of 2 different heights, 15 cm and 38 cm, provides some insight into why the relative advantage of *C. ariakensis* may change among sites even with salinity held constantly high. Varying elevation off the bottom had no detectable impact on growth of *C. gigas* and did not change the survivorship advantage that *C. gigas* held over both other oysters. However, culturing *C. ariakensis* on the bottom consistently reduced its growth rate. Growth of *C. virginica* also exhibited lower growth in bottom culture at 1 of the 2 sites, but the native oyster was sufficiently less sensitive to the bottom environment such that the statistically significant growth advantage held by *C. ariakensis* over *C. virginica* at both rack elevations disappeared on the bottom at one site. Similarly, *C. ariakensis* held a detectable survivorship advantage over *C. virginica* only on high racks. Because the concentration of suspended sediments decreases dramatically with elevation in the water column in estuaries and suspended sediments can interfere with suspension feeding (Rhoads & Young 1970), our results from the manipulation of elevation of culture imply that *C. ariakensis* is more sensitive to elevated turbidity than the other two oysters. Consequently, variation in suspended sediment load may help explain the high variation in *C. ariakensis* survivorship among high-salinity sites. Such sensitivity to bottom culture implies that *C. ariakensis* may experience difficulty in becoming established in more turbid regions of estuaries.

Although results of our manipulation of culture elevation imply greater sensitivity of *C. ariakensis* to turbidity, it is doubtful that this explanation accounts for all the variation in its survivorship among high-salinity sites. Other factors varied among sites, such as extensive shell fouling by barnacles and tunicates at Newport River and Chadwick Bay, which may have contributed to mortality. Higher mortality rates could also be a consequence of the parasite *Bonamia* sp., which has caused extensive mortality among juvenile oysters in laboratory and field trials conducted at UNC-IMS (Bishop et al. unpublished data). Rearing organisms in hatcheries often results in extreme genetic bottlenecks (Gaffney et al. 1996, Launey & Hedgecock 2001), which could increase cultured species' susceptibility to parasites and diseases. In the absence of the ability to predict mortality from known independent environmental variables that could be measured *a priori* at any prospective aquaculture site, the possibility of high mortality renders culture of *C. ariakensis* in high salinity a very risky proposition.

Creating any structures rising more than 15 cm off the bottom in North Carolina waters requires growers to obtain a water column lease in addition to the standard bottom lease. Because water column leases historically have been very difficult to obtain in North Carolina and cost an additional \$100 per acre, advantages of using high racks must outweigh the added expense. Elevating oyster racks from 15 to 38 cm increased oyster growth and survivorship of *C. ariakensis* at both sites, growth of *C. virginica* at Newport River, and survivorship of *C. virginica* at both sites. The magnitude of the effects of increasing rack height from on the

bottom and at 15 cm to 38 cm was greatest for *C. ariakensis*, whereas elevating oysters to 38 cm did not affect growth or survivorship of *C. gigas* at either site. Elevating oysters from the bottom to 15 cm generally did not affect growth or survivorship for any of the 3 species. Our results suggest that oyster growers culturing *C. virginica* or especially *C. ariakensis*, but not *C. gigas*, might consider obtaining a water column lease, though a complete bioeconomic evaluation of whether increased growth and survivorship outweigh the additional costs should be considered first.

Our results from trial culture comparing the performance of 2 non-native oysters to the native eastern oyster provide reasonably clear conclusions. *C. gigas* consistently outperforms both other oysters in growth and survivorship in high-salinity waters, but does less well than the other 2 oysters in intermediate salinity. At high salinity, *C. ariakensis* can suffer extremely high mortality, perhaps in part from exposure to high turbidity or the parasite *Bonamia* sp., but the environmental determinants are not well enough known to predict where survival will be good or bad. Consequently, on the basis of unpredictable and occasionally massive mortality, culturing *C. ariakensis* at sites of high salinity is risky. On the other hand, *C. ariakensis* has a substantial growth advantage over both of the other 2 oysters at intermediate salinity and likely also has a survivorship advantage. The range of viable salinities for successful culture of *C. ariakensis* does not extend below approximately 10‰; however, in the range of 15‰ to 25‰, this oyster grows faster and suffers less from the oyster diseases that plague *C. virginica*. Elevation of *C. ariakensis* during culture should be a viable strategy to increase survivorship and growth, but the cost-benefit ratio of obtaining water column leases should be examined further in North Carolina. Incorporation of all these results into a bioeconomic model is now necessary to quantify and compare the additional value that could be generated from higher growth and/or survivorship rates of non-native species relative to the risks associated with introducing a non-native species.

Because oyster consumers in eastern North Carolina prefer *C. virginica* over either non-native species when consumed raw and *C. gigas* when eaten cooked (Grabowski et al. 2003), differences (if any) in the price of each species for both raw and steamer markets must be incorporated into economic evaluation of the profitability of culturing non-native oysters. Given that the added cost of producing triploid oysters to avoid wild introduction is high and non-native oysters may be somewhat less palatable, culture of triploid non-native oysters may prove economically non-viable. Making non-native oysters available for aquaculture may, however, lower the probability of unsanctioned and uncontrolled introduction of reproductively capable non-native oysters into the environment (National Research Council 2003), thereby reducing the risk of potentially dire ecologic impacts of introducing a non-native species (i.e., competition with native species, unintentional introduction of additional predators and/or diseases, etc.). Finally, potential ecosystem benefits (i.e., water filtration, habitat provision) of promoting bivalve aquaculture should also be considered in deciding about permits for culturing non-native oysters. The question of whether to attempt to establish breeding, self-replicating populations of a non-native oyster entails consideration of many more issues, but requires more biologic information on potential risks versus economic and ecosystem benefits.

ACKNOWLEDGMENTS

The authors thank Matt Kimble, Chris Stewart, Christina Tal-
lent, and Rachael Wagaman for assistance in culturing the oysters

in the field and conducting laboratory assays. Stan Allen, Jr., of the Virginia Institute of Marine Sciences provided disease-free triploid seed for both non-native oysters and much guidance. The authors thank Joe McClees, Carla Gwaltney, and numerous other supporters. This manuscript was greatly enhanced from comments by Sandy Shumway and two anonymous reviewers.

Mack Salter and Brian Sheppard also contributed their knowledge as commercial shellfishermen. This research was supported by the North Carolina General Assembly (through the Rural Development Foundation and the Fishery Development Foundation) and the North Carolina Department of Environment and Natural Resources.

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REPRODUCTIVE CYCLE AND MORTALITY OF THE JAPANESE OYSTER *CRASSOSTREA GIGAS* CULTURED IN BAHÍA FALSA, BAJA CALIFORNIA, MÉXICO

JORGE CÁCERES-MARTÍNEZ,* SERGIO CUIEL RAMÍREZ GUTIÉRREZ,
REBECA VÁSQUEZ-YEOMANS, AND PATRICIA MACÍAS MONTES DE OCA

Laboratorio de Biología y Patología de Organismos Acuáticos del Departamento de Acuicultura, Centro de Investigación Científica y de Educación Superior de Ensenada, Apdo. Postal 2732, 22860, Ensenada Baja California, México

ABSTRACT The gonadal development by estimating the percentage of cell components of the mantle (MC) of cultured oysters (*Crassostrea gigas*) and mortality were studied in the Bahía Falsa, B. C. México from May 1996 to April 1997. Two localities, inner and outer zones of the Bay, were considered. Oysters were grouped in accordance with their position in the culture art, upper, middle, and lower level. Differences among the percentage of developing gametes (DG) and ripe gametes (RG) were according to the locality of the Bay and the position of the oysters in the culture art. In the outer locality, ripe gametes started to appear in February and reached their maximum proportion in May and June (around 60%). There was a trend of minor percentages of DG and RG in the lower level. Reproductive activity extends to November in the upper and middle levels. From September to January the no reproductive period was in curse. In the inner locality the percentage of ripe gametes also started to appear in February but their percentages were higher (around 80%) in May and June than in the outer locality. The reproductive activity ends in September. Percentage of vesicular cells (VC) increased from June to January. There was minor percentage of DG and RG in the lower level. There was no relationship between the gonadic cycle and mortality of oysters but it was with the level of the culture art, and there was an increase in mortality values in the lower than in the middle and upper levels of the culture art and a major mortality in the outer than in the inner studied localities.

KEY WORDS: oyster, *Crassostrea gigas*, reproduction, gametogenesis, storage cells cycle, mortality

INTRODUCTION

The Japanese oyster *Crassostrea gigas* was introduced into several coastal lagoons of the Pacific coast of north west México, in 1973, including the Bahía Falsa, Baja California (Islas 1975). Nowadays the annual production in the region is approximately 1,622 mt with a value of 2.4 million dollars; approximately 1,800 employees are involved in this activity and Bahía Falsa contribute with 16% of these figures (Anuario estadístico de pesca, 2001).

The reproductive cycle of the Japanese oyster in Bahía Falsa was studied in the middle 1980s, when the raft culture was carried out (oysters remained underwater every time). Hernández-González (1989) studied oysters from one point in the inner area of the Bay and found that undifferentiated oysters are common in November, gametogenesis took place from March to June and spawning occurs from July to October. Paniagua-Chavez and Acosta (1995) studied two oyster populations in the middle and the outer areas of the Bay and found that spawning occurs from July to October in the outer area and from July to September in the middle area. Both studies were carried out using histologic analysis of the gonadal tissue. Currently the culture method used in the bay consists of wood frames, locally named "racas". Nylon ropes of 150 cm in length with attached oysters (named "sartas") are suspended from the racas (Fig. 1). Thus, oysters are affected by the tide; the oysters attached in the top of the line hang from the raca are exposed to air more time than those attached in the middle and bottom of the line. Moreover, food availability is also different in permanent submergence than in periodically exposed conditions. These differences may interact with the reproductive cycle of cultured oysters and have been recorded in other mollusks species like the blue mussel *Mytilus edulis*, *Mytilus galloprovincialis*, and *Mytilus californianus* from natural and cultured populations (Seed 1976, Ferrán 1991, Villalba 1995, Cáceres-Martínez & Figueras 1998, Curiel-Ramírez & Cáceres-Martínez 2004). The reproduc-

tive cycle is closely related with the meat content of oysters for harvest and the shelf life of the oysters, then knowledge of local variations in the reproductive cycle of oysters have a practical use for producers.

Some authors (Perdue et al. 1981, Beattie et al. 1988, Berthelin et al. 2000) have studied the probable relationship between gametogenic cycle and summer mortality in the Pacific oyster. The hypothesis of these studies is, after gametogenesis oysters became physiologically exhausted, and some mortality episodes can be related with that condition. Since 1998 some unusual mortalities have occurred in the Bahía Falsa, and the hypothesis of a pathogen agent involved has not yet been demonstrated (Cáceres-Martínez & Vásquez-Yeomans 2003, Curiel-Ramírez & Cáceres-Martínez 2004). Under the light of these findings, the study of the gametogenic cycle in cultured oysters exposed to different air exposition degrees during low tides and in different places of the Bay are relevant; moreover, the possibility that the gonadic cycle could be related with mortality episodes observed in the locality is also important.

This work was carried out to determine the gonadal cycle of the Japanese oyster *Crassostrea gigas* cultured at three different levels of the culture system and two localities in Bahía Falsa, Baja California, NW México and its relationship with mortality.

MATERIALS AND METHODS

The study was carried out in Bahía Falsa, Baja California, México from May 1996 to March 1997 (monthly samplings). Two localities, "Agromarinos" in the outer area of the bay and "Alfonsos", in the inner area of the bay were sampled (see Fig. 1). In each locality, one or two sartas carrying from 30 to 50 commercial size oysters were collected from the racas (mean total shell length, 12.73 ± 6.6 mm in Agromarinos; mean total shell length, 10.95 ± 3.1 mm in Alfonsos). The sartas, of about 150 cm in length, were divided into 50-cm sections: upper, middle, and lower levels (see Fig. 1). Approximately 10 to 20 oysters from each level were grouped separately for analysis. In Agromarinos the upper and

*Corresponding author. E-mail: jcaceres@cicese.mx

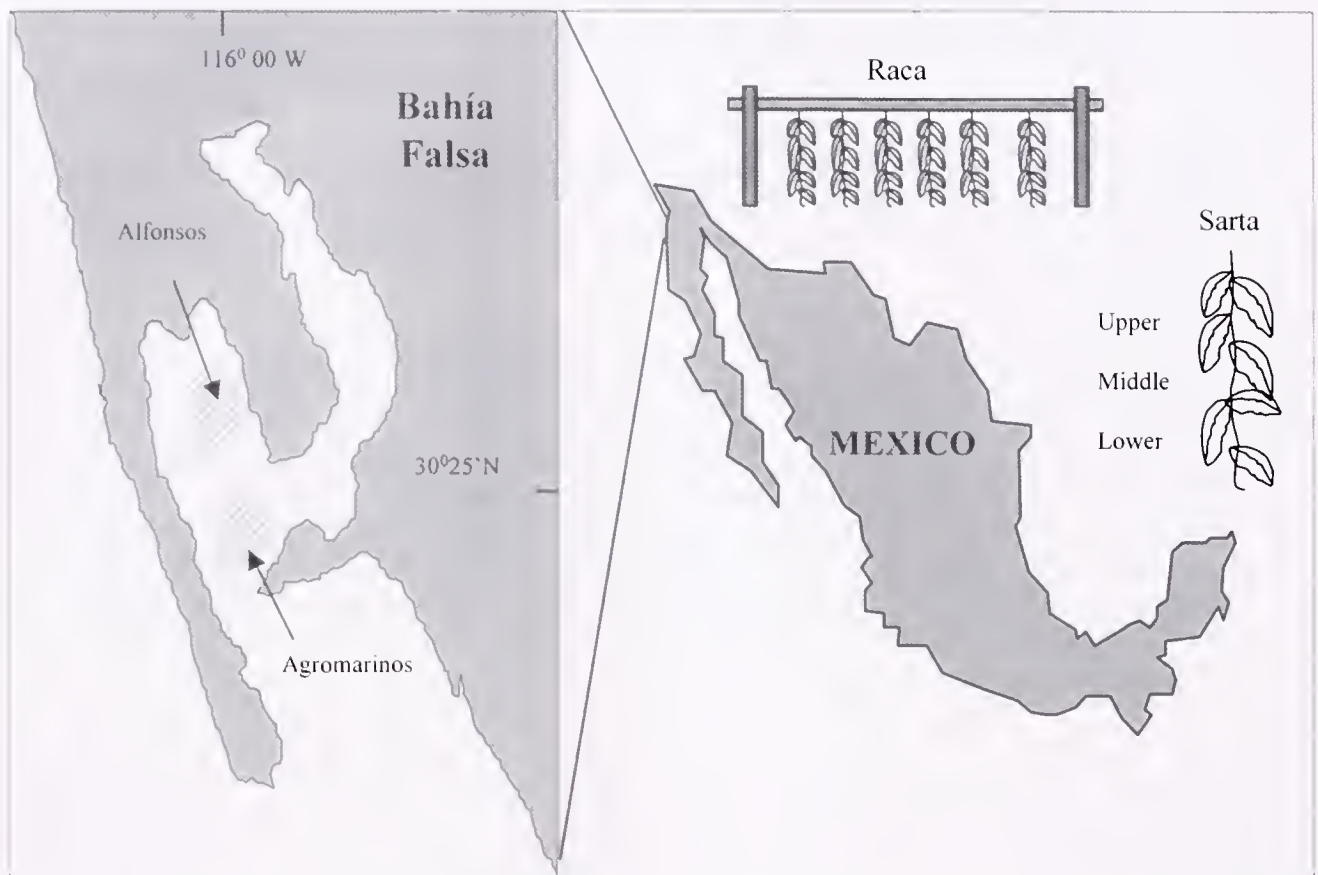


Figure 1. Map showing the inner (Alfonso's) and outer (Agromarinos) studied localities in Bahía Falsa, Baja California, Mexico. The oyster culture frames locally named "racas" and the nylon ropes where the oysters are placed named "sartas" are shown. For the study, oysters from the sarta were separated into three levels: upper, middle, and lower.

middle levels of the sarta are exposed to air during low tides, and the lower level of the sarta is always under water and never touches the bottom. In Alfonso's the entire sarta is exposed to the air during low tides and the end of the sarta touches the bottom on some occasions. During samplings, temperature ($^{\circ}\text{C}$) and salinity (ppt) in each locality were recorded with a conventional seawater thermometer and refractometer, respectively. Mortality was assessed by counting the number of empty shells from each sarta level every month, values showed cumulative mortality at each sarta level in the corresponding sampling month.

Live oysters were counted every month and after removing all fouling organisms with a brush and a jet of seawater, oysters from each level were measured (maximum axis as total length) and weighted (total wet weight). Oysters were opened and the meat was fixed in Davidson's fixative (Shaw & Batle 1957), and after 24 h an anterior transverse section was taken, including mantle tissue. Histologic sections with a thickness of $5\mu\text{m}$ were cut and stained with haematoxylin and eosin. The presence of gametes and storage cells were determined by quantitative sterology using a Weibel (1969) graticule, following the method described by Lowe et al. (1982). Briefly, five random counts were performed on each histologic slide. Results are given as a percentage of mantle volume occupied by the gamete development stages and cell types and defined as follows: ripe gametes (RG) in the female: it is the stage in which oocytes are free within the follicles and some oocytes remain attached to the follicle wall; whereas in the male: it is the stage when follicles are filled by spermatozoa arranged in

characteristic bands. Developing gametes (DG) in females: when rounded oocytes along with oocytes are attached to the follicle wall; whereas in the male: when varying quantities of developing spermatogenic cells are present. The presence of hemocytes around gametes and conditions of the follicle walls were recorded to determine if gamete reabsorption was occurring. The other cell components in the mantle were: vesicular cells (VC). Empty follicles (EF) were recorded when the points of the graticule indicate an empty area inside the follicle.

Differences between cell types (RG and DG) in the mantle volume per level of the sarta in each locality were assessed by a nonparametric Kruskal-Wallis ANOVA by Ranks test. Two way-ANOVA tests were used to compare significant differences of cell types in the mantle volume of *Crassostrea gigas* between localities. Student's *t*-test was used to compare significant differences between oyster mortality from Agromarinos and Alfonso's. A correlation between mortality and cell types (RG and DG) was carried out. Salinity and temperature were also compared with this statistical test.

RESULTS

Variations in percentage of the different cell types in the mantle volume of *Crassostrea gigas* from Agromarinos are shown in Figure 2. Presence of RG in the upper and middle level was recorded from February to November. In the lower level RG were observed from February to March and August. Reproductive ac-

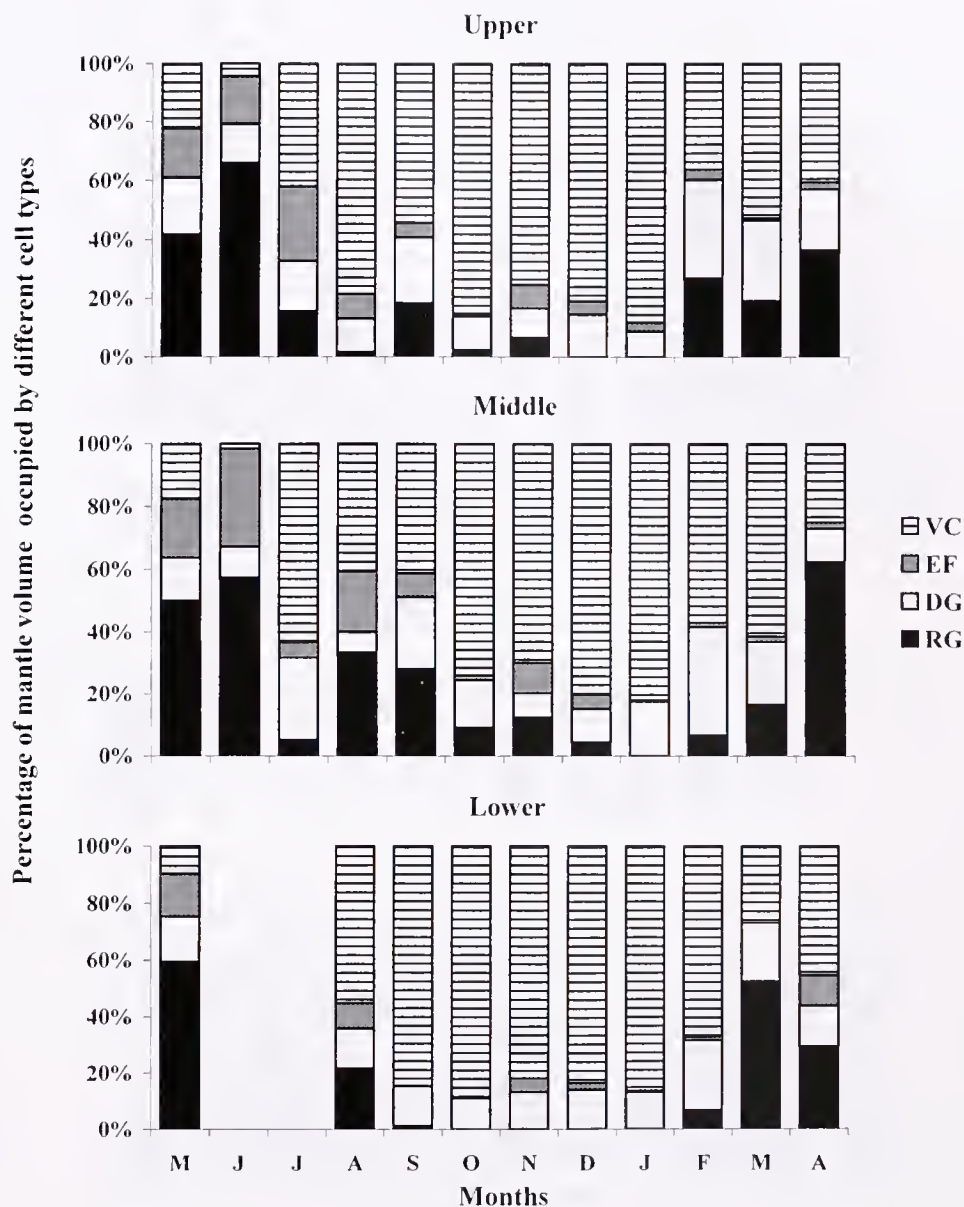


Figure 2. Variations in percentage of the different cell types in the mantle volume of *Crassostrea gigas* from different levels of the sarta of the outer locality, Agromarinos.

tivity extends to November in the upper and middle levels. Reabsorbing conditions were similar in the upper and middle levels of the sarta. This activity seems to be different from those observed in the lower level, where the no reproductive season extends from September to February (VC reach values up to 70%). However, these differences were not statically significant ($H = 1.7$, $P = 0.89$). Samplings from June and July in the lower level were lost.

Variations in percentage of the different cell types in the mantle volume of *Crassostrea gigas* from Alfonsos are shown in Figure 3. In general, these variations show that the gametes maturation starts in February, reaching its maximum levels in May and June. Afterwards, there is a period of reabsorption of gametes, indicating the end of reproductive season in September. The no-reproductive season starts with the reabsorption of gametes in June continuing until February when the reproductive period starts. During that

time VC are in high percentages (up to 70%). These variations were very similar between the upper and middle level of the sarta, but the presence of RG and reabsorbing conditions in the lower level was minimal, these differences were significant ($H = 12.7$, $P = 0.02$). Samples from all levels of the sarta were losing in April.

A comparison of variations in percentage of the different cell types in the mantle volume of *Crassostrea gigas* between localities suggest that reproductive conditions (presence of RG and DG) are more frequent in the upper and middle levels from Agromarinos (outer locality) than in Alfonsos (inner locality) (see Figs. 2 and 3), and these observations were statistically significant ($F = 4.3$, $P = 0.03$).

In Agromarinos, the highest mortalities were recorded in July and March. There was a trend of higher mortality in the lower and middle levels, but not in the upper level of the sarta (Fig. 4A). In

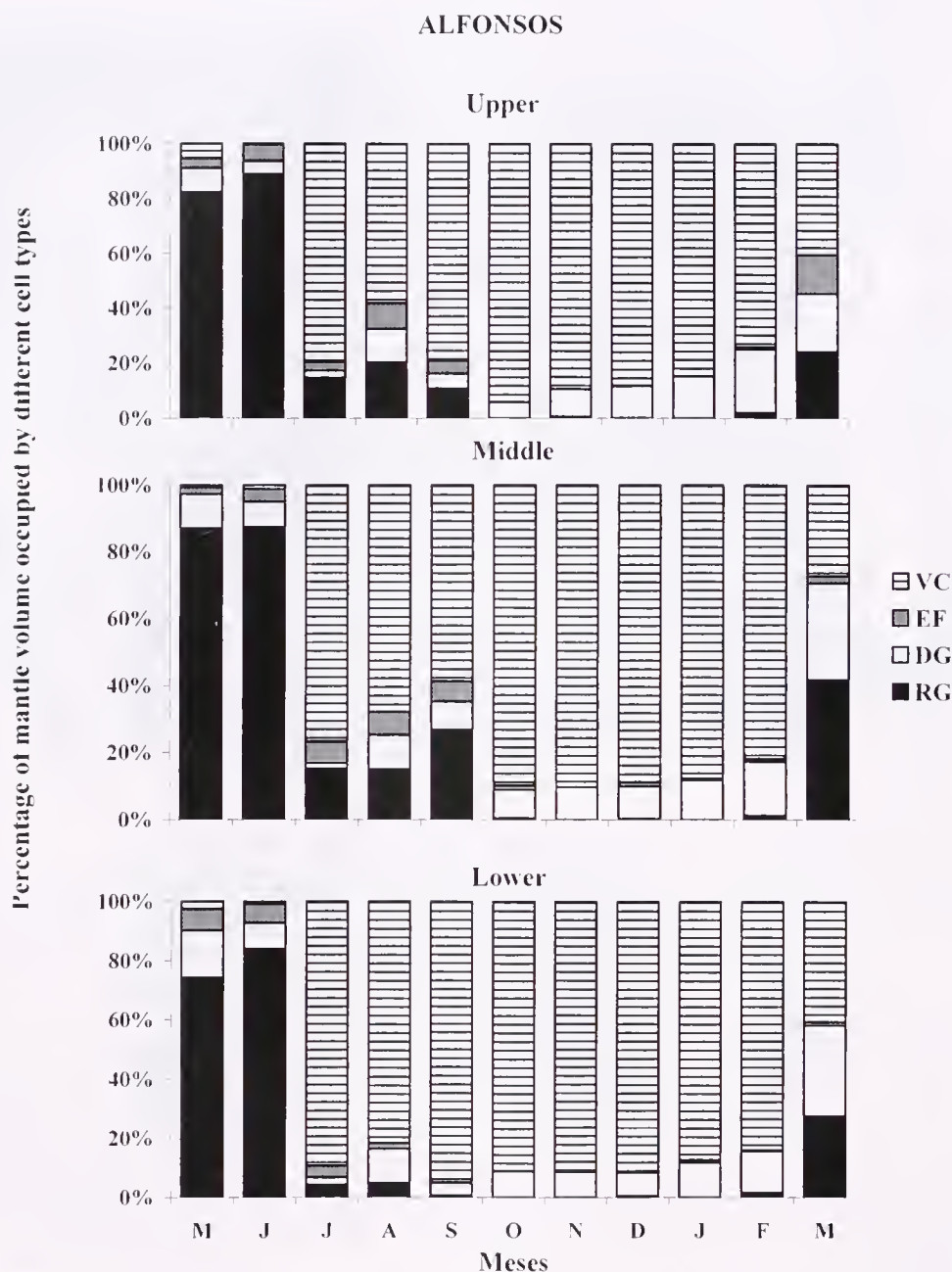


Figure 3. Variations in percentage of the different cell types in the mantle volume of *Crassostrea gigas* from different levels of the sarta of the inner locality, Alfonso's.

Alfonso's, the highest mortalities were observed in February and March. The highest mortalities were observed in the lower level (see Fig. 4B). There was no correlation between the presence of RG and DG and mortality ($r = 0.09276$, $P > 0.05$). Mortality was higher in Agromarinos than in Alfonso's ($t = 2.54$, $P < 0.05$). There were no significant differences between mean temperature and salinity from both sample sites and their values exhibited similar monthly variations (t -test, $P > 0.05$) (Fig. 5).

DISCUSSION

A general scope of view of reproductive cycle in both localities and levels of the culture art, showed a reproductive season from

middle winter to early summer. The maximum reproductive period was from middle to late spring. This pattern was close to those recorded by Hernández-González (1989) and Paniagua-Chavez and Acosta Ruiz (1995), these authors found a reproductive season from late winter to summer. Interannual differences in the reproductive cycle of the same species in the same locality may be related to fluctuations in the environmental conditions occurring in a particular year (Seed 1976).

A particular analyses of the studied reproductive cycles suggested that, differences in percentage of MC between the outer and the inner locality could be related to differential food availability. Lara-Lara and Alvarez-Borrego (1975), Millán-Núñez et al. (1982), and González (1997) found that chlorophyll was more

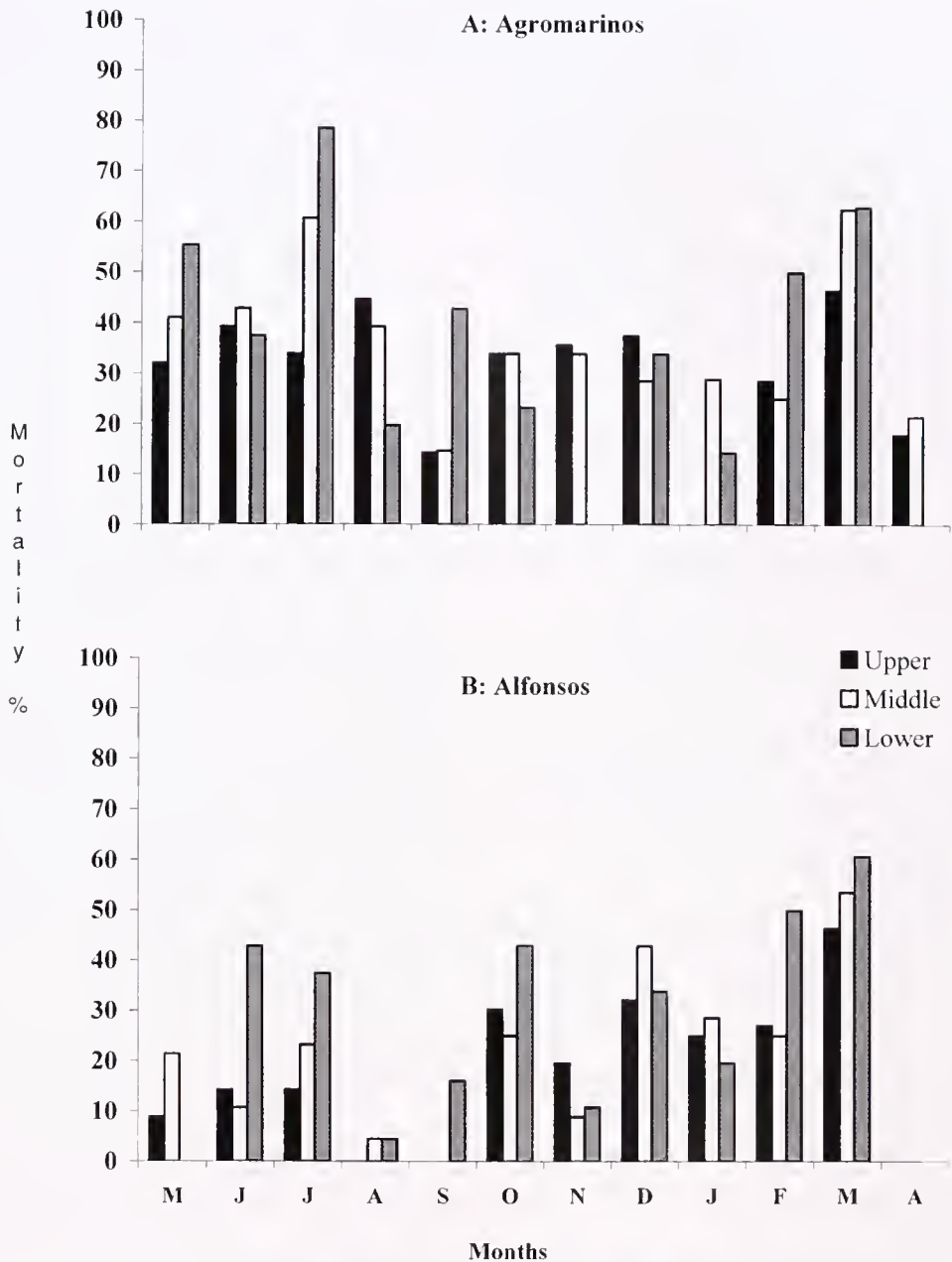


Figure 4. Percentage of mortality of *Crassostrea gigas* in, (A) the outer locality, Agromarinos and, (B) the inner locality, Alfonsos; at 3 levels in Bahía Falsa, Baja California, México.

abundant in the outer than in the inner part of Bahía Falsa. García-Esquivel et al. (2000) and García-Esquivel et al. (2003) found that shell growth rates were 1.5 times higher at the outer than the inner of the Bay. Thus, oysters from the outer area are placed in a food rich area, which could result in more energy available to gametogenesis, and, in consequence a more reproductive activity in contrast to the inner area. Statistical comparison of DG and RG of oysters from the three levels of the sarta in outer locality indicates that there are no differences. Placing loose samples during 2 months in the lower level of the sarta could affect that conclusion. The trend of minor reproductive activity in the lower level of the sarta seems apparent (see Fig. 3). In the inner locality, differences in the percentage of DG and RG indicate that reproductive activity is minor in the lower level of the sarta than in

the middle and upper levels, and in this case this is statically confirmed. This reduction in the reproductive activity could be related to micro environmental conditions. Detritus produced by the oysters has resulted in a muddy bottom where organic degradation is common (Villareal-Chávez 1993). These conditions could be detrimental for the oyster gametogenesis. In contrast, it seems that environmental conditions related to air exposition during low tides (i.e., temperature changes, food availability, desiccation) of upper and middle levels have a similar influence on the reproductive activity of the oysters placed at these levels of the sarta.

Contrary to the observations of Perdue et al. (1981), Beattie et al. (1988), and Berthelin et al. (2000), mortality was not related to the reproductive condition, high mortality values in the outer locality

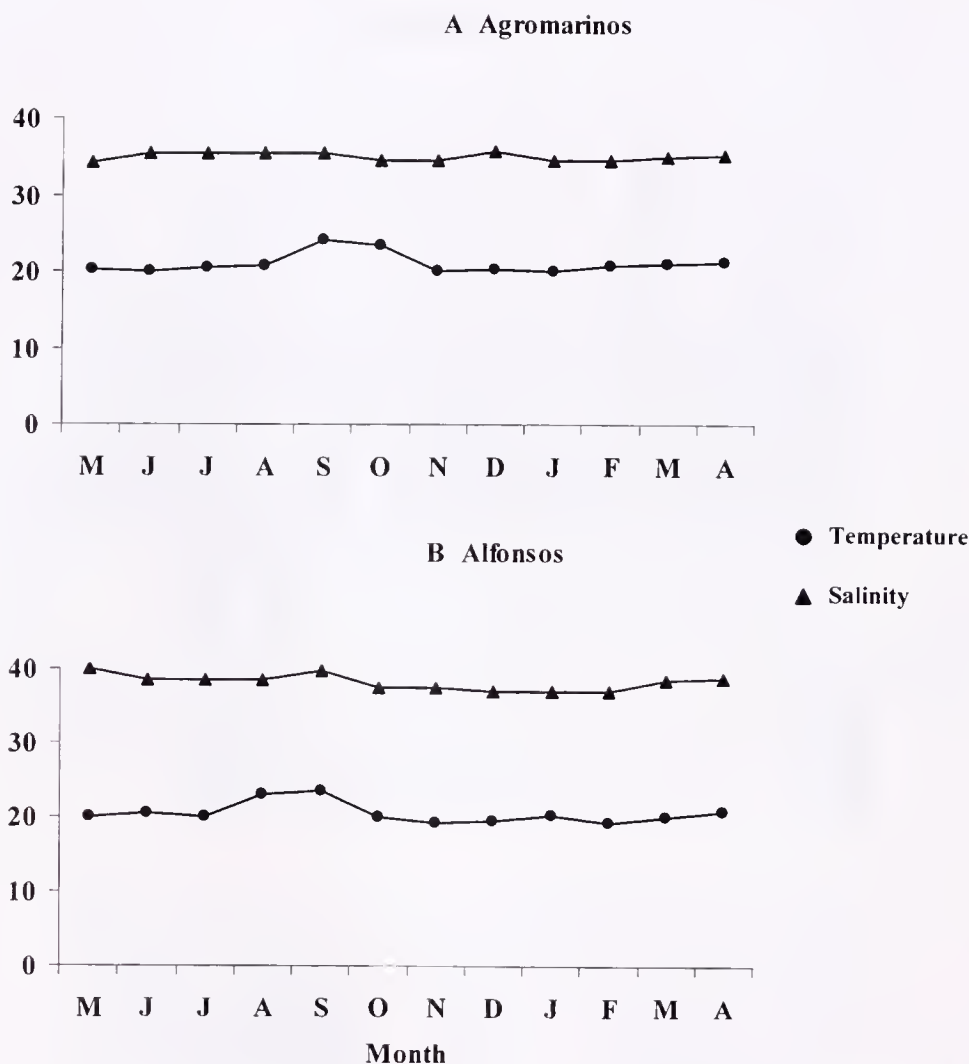


Figure 5. Fluctuations of temperature ($^{\circ}\text{C}$) and salinity (ppt) in the 2 localities studied, (A) the outer locality, Agromarinos and; (B) the inner locality, Alfonsos in Bahía Falsa, Baja California, México.

took place in July and March, the end and starting of the reproductive season respectively. In the inner locality, highest mortality rates were observed in February and March at the start of the reproductive season. However, the observed mortality tendency (highest mortality values in the lower level of the sarta) in the outer and inner localities suggests that muddy bottom where organic degradation occurs (Villareal-Chávez 1993), could be related to oyster survival. Detailed studies on mortality and physiologic condition of oysters are needed.

The major mortality values recorded in the outer than in the inner locality could be related to the fact that the inner locality is located near a tide channel where the water current is fast and may favor renewal or exchange of water near the bottom (Gosline & Stewart 1962). In contrast, the outer locality is placed near beds of sea grass (*Zostera marina* L.), where water exchange is low (Gosline & Stewart 1962). The high cumulative mortality values recorded reinforce the hypothesis of a possible pathogen as a causal

agent (Cáceres-Martínez & Vásquez-Yeomans 2003, Vásquez-Yeomans et al. 2004). Salinity and water temperature values were similar in both sampling sites and they are considered as appropriated for Japanese oyster growth and survival (Imai 1982, Pauley et al. 1988, Kennedy et al. 1996).

Usefulness of this information is related to the condition of the oyster for market, high meat content suggest harvest time; however, very mature animals may spawn during marketing, reducing their shelf life. High mortality values in the lower level of the sarta, suggest that placing sartas near the bottom should be avoided.

ACKNOWLEDGMENTS

To Alfonso, R. García and V. Guerrero for their assistance during sampling, Y. Guerrero and E. Gorrostrieta for help in data processing and graphics. This work was supported by CICESE project 623106 and CONACYT 225080-5-3933PB.

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A COMPARISON BETWEEN A SUCTION DREDGE AND A TRADITIONAL OYSTER DREDGE IN THE TRANSPLANTATION OF OYSTERS IN DELAWARE BAY

ERIC N. POWELL AND KATHRYN A. ASHTON-ALCOX

Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Ave.,
Port Norris, New Jersey, 08349

ABSTRACT One mechanism to enhance oyster production is the timely transplant of oysters from nursery beds to beds used for commercial harvesting. Transplanting in Delaware Bay is normally done with a traditional oyster dredge. Such dredges can concentrate market-size oysters, a desirable characteristic for some transplant goals. Unfortunately, catch rates are slow. The suction dredge is much faster, but suction dredges likely do not concentrate large oysters and, by removing most surficial material, may reduce bottom shell coverage and decrease bottom complexity. We investigated the relative benefits of using a traditional oyster dredge and a suction dredge in a transplant program. In this study, traditional oyster dredges used for transplant operations had dredge efficiencies of approximately 5%, about 100 bushels of material being loaded per hectare swept. The tendency for the dredge to catch larger particles preferentially was negated by the tendency to operate the dredge at below-optimal efficiencies. Nevertheless, deck loads contained a factor of 2 to 3 more oysters per bushel than present on the bottom. The suction dredge operated very differently, although deck loads contained 1 to 3 times as many oysters as were present on the bottom. Catch efficiencies were high, between 19% and 58%. Swept area per bushel loaded was much lower, about 600 bushels being loaded per hectare swept. Catch efficiencies were highest for small particles. Dredge efficiency rose markedly after transplanting, from 6% to 28% on the plots worked by the traditional oyster dredge and from 11% to 56% on the plots worked by the suction dredge. Nevertheless, neither method proved deleterious to bottom complexity, cultch availability, oyster growth and mortality, or population health. In a sustainable transplant, the number of small oysters and amount of cultch moved should be minimized. This goal was not achieved. The suction dredge, by selective removal of smaller particles enriched in juveniles and cultch, risks a long-term decline in live oyster abundance and shell coverage. The traditional oyster dredge has the inherent capability of concentrating larger animals, but, as used in the transplant process, much of the selective advantage disappears. A behavioral shift to exploit the desirable selective advantage of the traditional oyster dredge may improve the efficiency of the transplant program.

KEY WORDS: oyster, *Crassostrea*, dredge efficiency, transplantation, fisheries management, Delaware Bay

INTRODUCTION

One mechanism to enhance oyster production is the timely transplantation of oysters from nursery beds to beds used for commercial harvesting (Owen 1953, Jory & Iversen 1985, Powell et al. 1997, Kraeuter et al. 2003). In Delaware Bay, nursery beds are in lower salinity water where lower predation rates and lesser disease prevalences increase oyster survivorship. Unfortunately, oysters frequently do not reach market size in harvestable numbers on these beds or, if they do, fail to achieve market standards of meat quality (Powell et al. 1997, Ford 1997, Kraeuter et al. 2003). Transplantation of these oysters downbay, however, normally results in a rapid increase in meat weight (condition) as well as increased growth (Kraeuter et al. 2003). Timed appropriately, such oysters can become marketable before the increased disease and predation pressure downbay markedly reduce their numbers. However, an efficient transplanting scheme would select from the nursery beds only oysters that can become marketable in one growing season, reserving on the seed beds smaller oysters requiring multiple growing seasons to achieve marketability; thereby minimizing loss to predation and disease.

In Delaware Bay, transplanting is normally done with a traditional oyster dredge. The dredge used by nearly all boats is a 24-tooth dry dredge, with tooth length approximately 44 mm, a mouth opening of 1.27 m × 51 cm, and a bag consisting of 17 rows of 50.8-mm rings (Fig. 1 in Powell et al. 2002). Dredge efficiency can exceed 50% (Powell et al. 2002), but is normally much lower (Chai et al. 1992, Powell et al. 2002), particularly when used during commercial harvesting (Banta et al. 2003). The dredge is less efficient for smaller oysters and much less efficient for cultch (Powell et al. 2002). A culling machine, an on-deck rotating drum

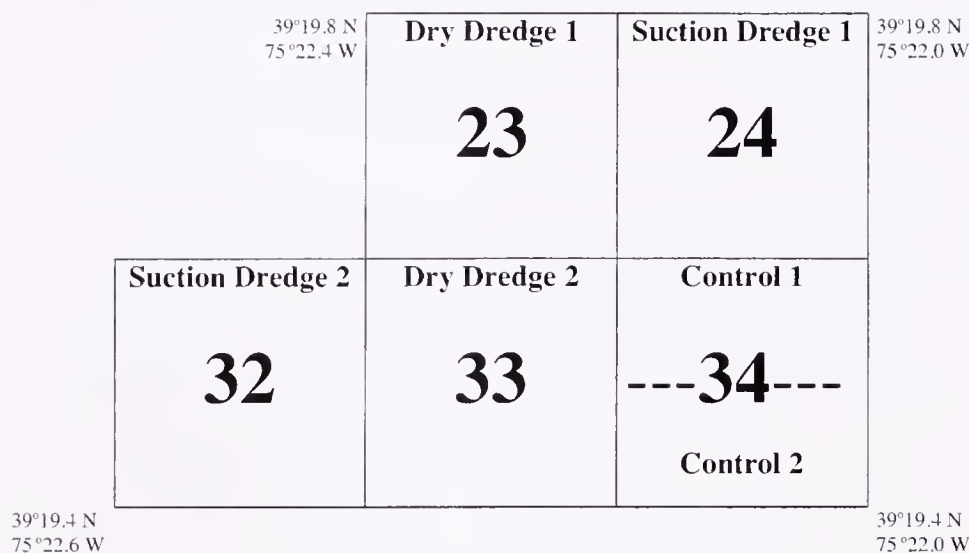
with bar spacing set to retain only the larger oysters and shell, can increase the concentration factor for market-size oysters (HSRL 2003). Hence, use of the traditional dredge and a culling machine should minimize downbay transport of small oysters that require more than one growing season to achieve market size.

Unfortunately, catch rates for dry dredges are slow. The average oyster boat moved 900 bushels[†] of oysters per day during the 2003 transplant program in Delaware Bay (New Jersey Department of Environmental Protection [NJDEP], pers. comm.). An alternative is the suction dredge. The suction dredge is essentially a large vacuum cleaner. Water is pumped through a hose from the bottom to the deck. As a consequence, the surface material under the dredge is vacuumed up through the hose to the deck, washed over a fine-mesh sorter to remove mud and sand, and then deposited on the deck. A suction dredge of 2.44-m swath can easily move 3,500 bushels in the time the dry dredge moves 900 bushels.

Suction dredges are often used to clean fouled shell from leased grounds, to move spatulated cultch after shellplanting, or to remove predators (Ismail 1985). In these activities, the objective is to remove virtually 100% of the surficial material. However, the transplantation of oysters downbay from nursery grounds must be done sustainably. Suction dredges, by removing most surficial material, may remove more cultch and small oysters than dry dredges (e.g., Ismail 1985), consequently reducing shell coverage on the bottom and, hence, bottom complexity, while transplanting small oysters downbay that will not survive to be marketed. On a per-bushel basis, the economic cost of using dry dredges is high, however; on this basis, a suction dredge might be the preferred tool

[†] A standard New Jersey bushel is 37 qt or 35 L.

COHANSEY GRIDS



NEW BEDS GRIDS

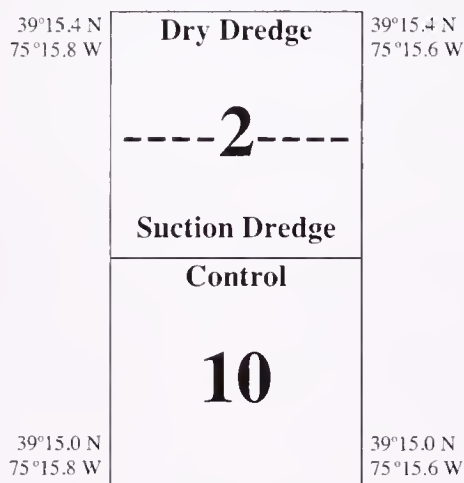


Figure 1. Location of transplant grid plots within the HSRL survey grid system (Fegley et al. 1994).

for transplanting. Accordingly, we carried out an investigation to determine to what degree use of a suction dredge, rather than a dry dredge, in transplanting might negatively impact the oyster population on the nursery bed, the anticipated long-term harvest from transplanting activity, and bottom habitat complexity.

METHODS

Experimental Design

Six contiguous experimental plots oriented in a 3 × 2 array were demarcated on Cohansey Bed in Delaware Bay (Fig. 1 in Banta et al. 2003). This bed historically has been an important nursery bed. Presently, oyster abundance on this bed and neighboring beds is at or above the 75th percentile of abundances recorded since 1989 (HSRL, 2003) when Dermo disease caused by the endoparasitic protozoan *Perkinsus marinus* first became epizootic in Delaware Bay (Ford, 1996). Two of these six plots,

HSRL survey grid plots 23 and 33 (Fig. 1, see also Fegley et al. 1994), each 0.2°-latitude × 0.2°-longitude or 26.3 acres (10.63 ha) in size, were assigned, one each, to 2 dry-dredge boats, the *F/V Mabel Hollinger* and the *F/V Robert Bould*. Two plots of the same size, HSRL survey grid plots 24 and 32 (Fig. 1, see also Fegley et al. 1994), were assigned to a suction dredge boat, the *F/V Jeanne Christine*. The final two plots, each 0.1°-latitude × 0.2°-longitude subdivisions of HSRL survey grid plot 34 (Fig. 1), of 13.15 acres, were designated unfished controls.

A preliminary survey of the four experimental plots was conducted by a vessel of known dredge efficiency (Powell et al. 2002), the *F/V Howard W. Sockwell*, on May 9, 2002. In New Jersey, the number of oysters transplanted yearly from the nursery beds normally has been limited to 10% of the standing stock ≥63.5 mm in size (HSRL, 2003). We chose, in designing the experiment, to exceed this goal on experimental plots by about a factor of 2 so as to evaluate the effects of a suction dredge at a level of impact

unlikely to occur routinely in the transplant program. Thus, 20% of the total number of ≥ 63.5 -mm oysters within each grid plot was targeted for transplant. Accomplishing this exceeded the allocated 1-day for each of the dry-dredge boats, but took less than 1 day per plot for the more efficient suction dredge. Oysters and cultch put on deck were transplanted downbay to New Beds HSRL survey grid plot 2 (Fig. 1 in Banta et al. 2003, see also Fegley et al. 1994). The suction dredge boat transplanted to a single 0.1°-latitude \times 0.2°-longitude section of this plot. The two dry-dredge boats transplanted to a contiguous section of the same size. An abutting plot to the south, HSRL survey grid plot 10, was used as a control.

The volume of material moved downbay by each boat was estimated geometrically. Detailed measurements of the accumulated material on deck were taken and the pile approximated as a series of prismatoids and cones that provided volumetric calculations from measurements of diameter and height at various locations.

Onboard Sampling During Transplanting

One-bushel samples were taken hourly for the dry dredge and half-hourly for the suction dredge during the dredging process. On the dry-dredge boats, paired samples were taken from the hopper, representing the material coming out of the dredge, and from the pile, representing material sorted for retention by the culling machine. This latter cull was transplanted downbay. On the suction dredge, a single bushel sample was taken because no culling occurred on deck.

Bed Sampling—Dredge

Each of the four experimental Cohansey plots, one of the Cohansey control plots (34B), and the New Beds experimental plot was sampled prior to the transplant on May 9, 2002, using the *F/V Howard W. Sockwell*. Each Cohansey experimental plot, both control plots, and both New Beds transplant plots were sampled soon after transplant on July 15, 2002, approximately 4 months later on November 5, 2002, and approximately 10 months later, on May 6, 2003, using the *F/V Howard W. Sockwell*. The New Beds control plot was sampled on May 6, 2003. During the approximately 10 months that elapsed between transplant and final sampling, the New Beds transplant was closed to fishing by the NJDEP. Banta et al. (2003) estimated that yearly swept area by the Delaware Bay oyster dredge fishery on New Beds equals about 101% to 123% of bed area; hence the need to close this experimental plot. The Cohansey plots were not closed because dredging by the Delaware Bay oyster fishery on these plots used nearly exclusively for transplanting has varied between 11% and 36% of bed area (Banta et al. 2003). Thus, little dredging was anticipated during the experimental time. Unfortunately, some removals may have occurred from the control plots during the oyster industry's late summer of 2002 transplant.

Each sampling time, three random 1-min tows were taken in each plot. Following transplanting, these samples were distributed randomly within the subsection of the plot from which most transplanted material was obtained. DGPS position was recorded every 5 seconds. All material caught by the dredge was volumetrically measured. A 1-bushel subsample from each dredge haul was retained for further analysis.

Bed Sampling—Diver

Divers were used to obtain quantitative samples prior to the transplant on June 10, 2002, just after the transplant on June 24,

2002, and about 10 months later, on April 28, 2003. Diver sampling was limited to the Cohansey plots because we were interested in the effect of dredging style on habitat complexity on the nursery bed. Initially, five sites were randomly chosen within each Cohansey plot. Following transplant, two of the five locations were resampled. The remaining three were randomly distributed within the area of the plot from which most of the transplanted material was obtained. A buoyed 23-m transect line was dropped from the *R/V Zephyrus* at each site. Twelve collection bags were affixed at equal intervals along this line. Divers were then deployed to retrieve all loose bottom material, including live oysters and boxes (dead, articulated valves) within a 0.25-m² quadrat haphazardly deployed at each collection site. The divers were instructed to take only the loosely consolidated material on the surface that would normally be taken by the dredge. In most cases, quantitative retrieval was simple because the consolidated portion of the bed was near the surface. In a few cases, unconsolidated shell extended downwards for some distance. In these cases, the diver took the upper portion of the shell until the collection bag was filled.

Laboratory Analysis

Diver quadrat samples and dredge-boat bushel samples were identically analyzed with three exceptions noted subsequently. Each bushel or quadrat sample was sorted into live oysters, boxes, cultch, and debris and the respective volumes measured. Debris includes such miscellany as rocks, gravel, and detached bionts such as sponges and erect hydroid/bryozoan colonies. Cultch was defined as single valves and shell fragments without attached live oysters or boxes. The assumption was that boxes represented recent mortality (Christmas et al. 1997). The longest dimension (anterior-posterior) of the right valve of each oyster and box >20 mm was measured. Live spat, oysters <20 mm, were tallied.

Clumps were defined as composites containing some combination of two or more shells, boxes, and/or live oysters. The 10 largest clumps in each bushel or quadrat sample, or all of the clumps if fewer than 10, were analyzed. The average number of clumps in a set of samples so derived is termed "minimum average" henceforth. The length and height of each chosen clump were measured. Maximum clump cross-sectional area (A) for these selected clumps was defined as: $A = \text{clump length} \times \text{clump height}$. The number of oysters, boxes, and spat per clump was enumerated.

Dry meat weights were taken for 20 oysters per bushel. Condition index was defined as:

$$CI = \frac{\text{dry weight (g)}}{\text{length (cm)} \times \text{height (cm)}}$$

For New Beds samples taken on May 6, 2003, 20 oysters per bushel were taken for *Perkinsus marinus* analysis following standard procedures (Powell & Ellis 1998).

Samples obtained during the transplant program onboard the *F/V Mabel Hollinger*, the *F/V Robert Bould*, and the *F/V Jeanne Christine*, were analyzed simply to estimate the number and size frequency of oysters present.

Estimation of Dredge Efficiency

Dredge efficiency, e , was estimated by comparing dredge samples of unknown efficiency to diver samples of known (100%) efficiency:

$$e = \frac{\left(\frac{\text{number of oysters or boxes (bushel)}^{-1} * \# \text{ bushels}}{\text{m}^2 \text{ dredge swept area}} \right)}{\left(\frac{\sum_{i=1}^{12} \text{number of oysters or boxes (diver sample)}^{-1}}{\text{number of diver samples} * 0.25 \text{ m}^2} \right)} \quad (1)$$

Because all comparisons were made on a 1-m² basis, the catchability coefficient q (Powell et al. 2002) was defined as the reciprocal of efficiency e :

$$a = \frac{1}{e}.$$

Sampling was carried out by diver and by the *F/V Howard W. Sockwell* using a traditional oyster dredge in survey mode. The term "survey mode" refers to the sampling protocol in which tows were of 1-min duration and catches never filled the dredge bag. Dredge efficiencies tend to be relatively high in this use (Powell et al. 2002). Estimates of dredge efficiency represent averages for each grid for each sampling time because diver transects were not conducted simultaneously with dredge-boat sampling and because diver transects were not oriented in or parallel to dredge-boat tow paths; thus, pairwise comparisons between a single diver transect and a single dredge-boat tow could not be made.

Statistical Analysis

For most analyses of dredge-boat samples, metrics, such as the number and volume of live oysters, were expressed on a per bushel basis rather than as a quantitative measure (m⁻²), because, in the case of bushel samples from the transplant boats, the bushel sample did not come from a known area and because transplanting is inherently a volumetric activity rather than an areal activity. Samples taken by the *F/V Howard W. Sockwell* were also quantitated using a correction for dredge efficiency obtained from quantitative diver samples and measured swept area from DGPS positions taken during the tow. Because of the limited number of dredge efficiency measurements and the scale of these corrections, significant differences between treatments must be provisional.

On the other hand, the use of volume standardization assumes an invariant cultch volume per bushel. Because cultch was not invariant, because live oysters comprised a significant fraction of the total volume, and because live oysters were preferentially removed in some treatments, statistics based on volume standardization also must be considered carefully.

The two sampling approaches, diver and dredge boat, each have their strengths and shortcomings. The dredge boat integrates a large area, but is not directly quantitative. The diver sampling is quantitative, but the smaller area sampled may be less representative of the total plot because dredging intensity during transplant was unevenly distributed and because of the inherent patchiness of oysters on the bed. Thus, we looked for trends that were sustained by both volume and quantitative standardization and for trends that were sustained by both dredge and diver sampling.

Most statistical analyses used ANOVA. Unless otherwise noted, all variables were ranked prior to analysis; thus most analyses are nonparametric (Underwood 1981, Sokal & Rohlf 1998). The experimental protocol for dredge sampling involved a nested series of samples, in that each Cohansey treatment had two replicate plots and each replicate sampling involved analysis of three 1-bushel samples per plot. Similarly, diver samples involve a nested set of 12 samples within each string, five strings nested

within each plot, and two plots per treatment. All ANOVA models were so designed.

Because no reason except proximity supported the assumption that control areas were representative of experimental areas, comparisons involving the effects of transplant were judged primarily by the significance of time \times location interaction terms. That is, we assumed equivalency of environment over the Cohansey (and New Beds) area used for our experiment, so that natural temporal changes should have been relatively uniform and, consequently, the time \times location interaction term would be insignificant without significant perturbation by the experimental manipulation of oyster transplant.

Whenever the desire was to conduct analyses on ratios of variables, such as condition index, the denominator was used as a covariate and the numerator as a dependent variable to avoid the increased variance that often accompanies ratios (Green 1986, Sokal & Rohlf 1998). Analyses evaluating the variance used the mean as a covariate because the variance normally increases with the mean (Elliott 1977, Green 1989). For each diver sample, we measured total volume collected and, as well, the volumetric contribution of shell, live oysters, boxes, and debris, to the sample. For tests in which volumetric subsets were dependent variables, the total volume was used as a covariate.

Size frequencies were compared using percentiles, normally the 25th, 50th, and 75th, and the mean size, to permit comparison with the main effects of time and treatment. Because the objective of transplant was to move market-size animals downbay, the size-frequency distribution was also investigated using the proportional contribution of small (<63.5 mm), submarket (63.5–<76.2 mm), and market-size (≥ 76.2 mm) individuals. Analyses evaluating proportional shifts among these 3 size groups used small or submarket abundance as the dependent variable and market-size abundance as the covariate.

The evenness in the distribution of dredging effort over the experimental plots was evaluated using Elliott's D (Elliott 1977):

$$D = \sqrt{\frac{\sigma^2}{\bar{x}} 2(n-1)} - \sqrt{2(n-1)-1} \quad (2)$$

where σ^2/\bar{x} is the variance-to-mean ratio. The metric used was the number of times the dredge was towed through each 0.01° latitude \times 0.01° longitude section of the plot.

RESULTS

Volume Transplanted and Concentration Factor

Between 445 and 1,504 bushels were transplanted from each experimental plot (Table 1). These values represent 10.8% to 27.1% of the standing stock of ≥ 63.5 -mm oysters estimated to be present prior to transplant. The suction dredge removed material more rapidly and so transplanted a higher fraction of the standing stock than the dry dredge in the allotted time (Table 1). The suction dredge boat also distributed its effort more evenly over the experimental plots (Fig. 3) than the dry-dredge boats (Fig. 2), although dredging effort was significantly contagiously distributed in all plots ($\alpha = 0.05$). Values of Elliott's D for the suction dredge were 22.2 for plot 32 and 33.8 for plot 24. Values for the traditional oyster dredge were much higher: 111.3 for plot 23 and 106.5 for plot 33. The increased and more unevenly distributed effort of the dry-dredge boats is in keeping with the standard operating procedure of these boats (Banta et al. 2003). Thus, the removal fractions

TABLE 1.

The number of bushels transplanted from each Cohansey plot, the fraction of the estimated oyster standing stock moved, the swept area covered during transplant operations, and the efficiency of extraction of live oysters from the bottom [i.e., equation (1)]. The catchability coefficient (q) is obtained as the reciprocal.

Treatment Plot	Bushels Transplanted	Fraction of Estimated Market-Size Abundance	Swept Area (ha)	Catch Efficiency
33 (Dry-Dredge)	445	11.6%	4.19	0.055
23 (Dry-Dredge)	608	10.8%	6.47	0.054
24 (Suction-Dredge)	1,504	15.8%	2.65	0.190
32 (Suction-Dredge)	1,131	27.1%	1.82	0.582

estimated in Table 1 are considerably higher in the areas concentrated upon, particularly by the dry-dredge boats, than for the plot as a whole. Dredge and diver sampling focused on these areas subsequent to transplanting.

We compared the number of oysters per bushel and their size frequency between the material brought onboard by the dry dredge

(in-hopper, Table 2) and the material subsequently culled by the culling machine and placed on the deck for transplant (on-deck, Table 2). The culling machine strongly selected in favor of ≥ 6 -cm particles (Fig. 4, Table 2). Culled material deposited on deck was enriched by a factor of 1.5 to 3.0 in these large particles relative to that brought onboard by the dredge (hopper-to-deck concentration factor, Table 2). Because many of these large particles are live oysters, on-deck culling concentrates submarket and market-size oysters. Live oysters loaded onto the deck for transplant ranged from about 1.5 to over 3.0 times more abundant per bushel of material than their proportion in diver samples taken directly off the bottom prior to transplant (bottom-to-deck concentration factor, unstandardized, Table 2). Thus, oysters were concentrated with respect to cultch. The size-frequency structure of oysters on the bottom and on the deck was about the same, however (bottom-to-deck concentration factor, standardized, Table 2), indicating that, in these two transplant experiments, the dredge tended to catch proportionately more small oysters while on the bottom and this counterweighed the culling machine's bias favoring larger oysters (Fig. 4).

The suction dredge operated in a distinctively different mode. Material brought on deck was underrepresented by large particles, including live oysters, relative to that on the bottom. Concentration

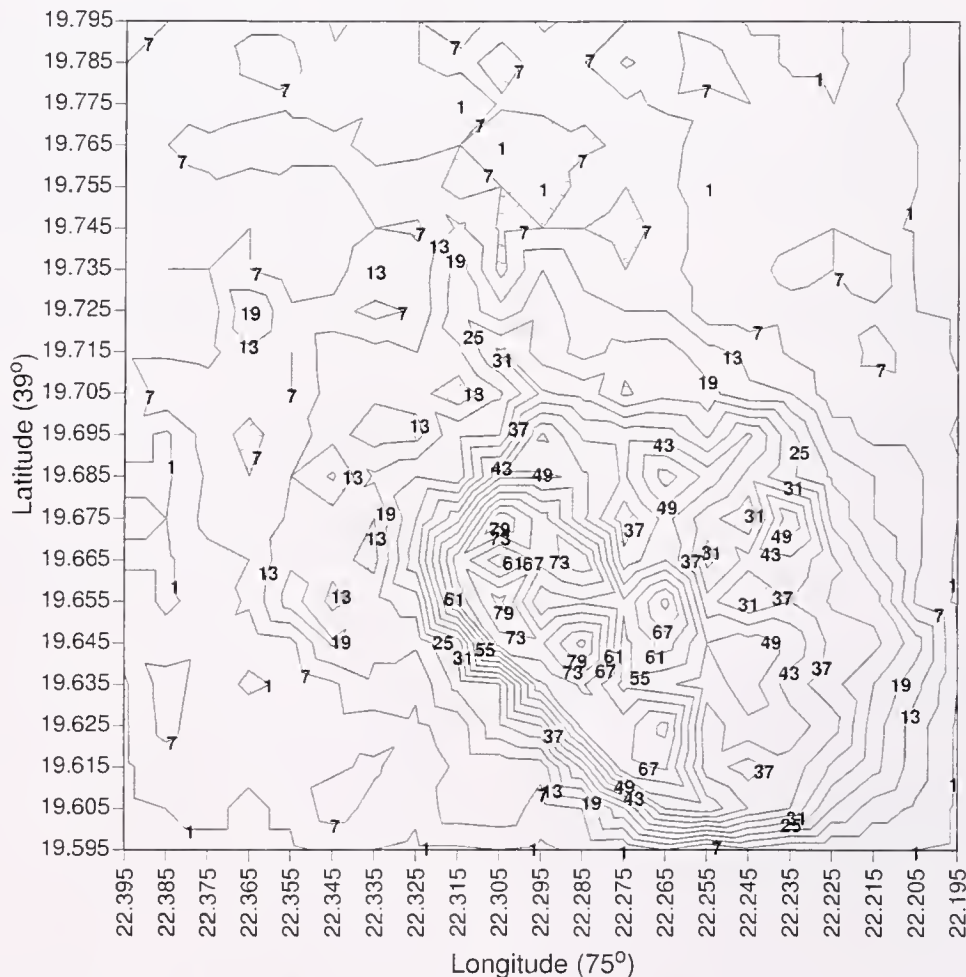


Figure 2. Contours of the frequency that an area was dredged during the course of transplanting from plot 23. The *F/V Robert Bould* carried out this transplant using a traditional oyster dry dredge. Contours are the number of times the dredge was taken through a 0.01° latitude \times 0.01° longitude rectangle, an area of 265.7 m^2 .

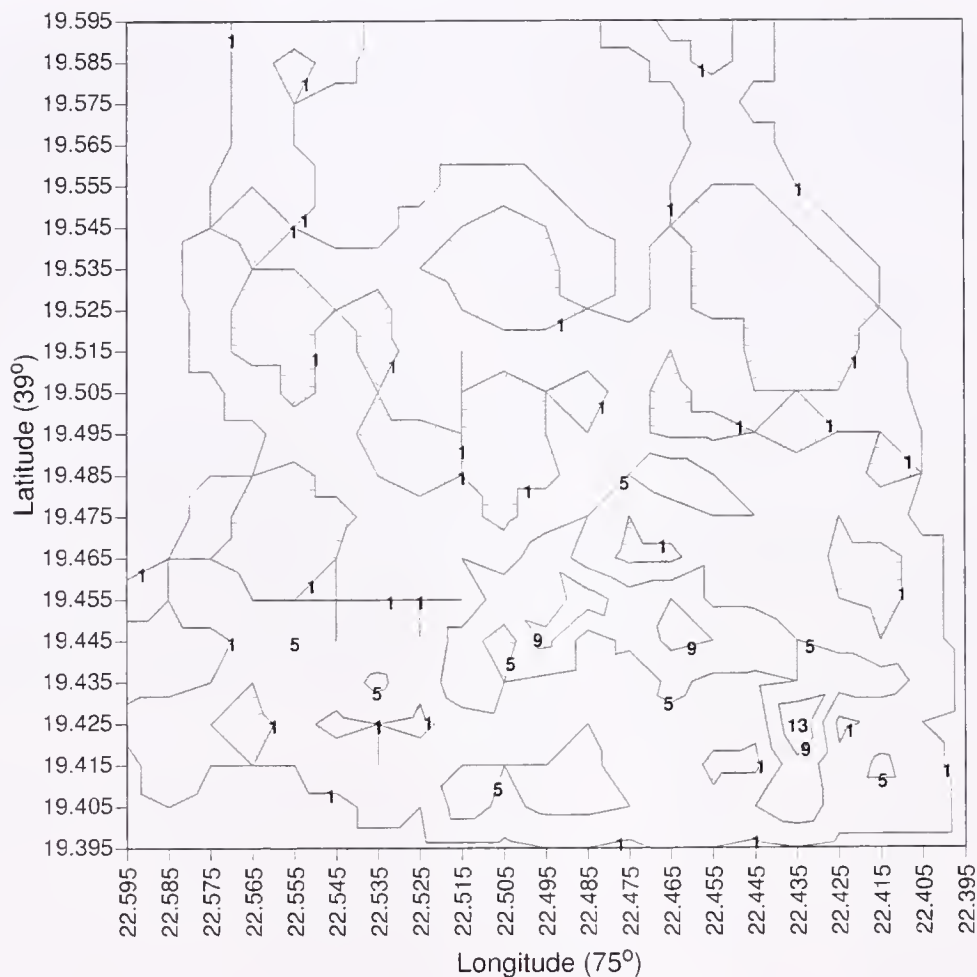


Figure 3. Contours of the frequency that an area was dredged during the course of transplanting from plot 32. The suction dredge boat, the *F/V Jeanne Christine* carried out this transplant. Contours are the number of times the dredge was taken through a 0.01° latitude \times 0.01° longitude rectangle, an area of 265.7 m^2 .

factors for oysters $\geq 6 \text{ cm}$ were often half or less of that observed for smaller oysters (Fig. 5, bottom-to-deck concentration factor, standardized, Table 3). The selection ratio between 4-cm and 7-cm oysters was 2.7 and 2.8 for the two transplants: the suction dredge was nearly three times as efficient in capturing 4-cm oysters as 7-cm oysters (Fig. 5). The equivalent values for the dry dredge were 0.9 and 0.8. The ratios of live oysters per bushel on the deck and on the bottom, 2.61 in one case (likely an overestimate—Table 3) and 1.16 in the other (Table 3), indicate that the suction dredge tends to concentrate live oysters relative to culch, albeit biased in favor of smaller individuals. Equivalent ratios for the 2 dry-dredge transplants, 2.64 and 2.46, averaged higher and the deck loads contained more large live oysters (Table 2). Note, in particular, that fewer of the largest oysters were loaded on deck by the suction dredge than were encountered on the bottom (bottom-to-deck concentration factor, unstandardized, Table 3). No size class was equivalently undersampled by the dry dredge (bottom-to-deck concentration factor, unstandardized, Table 2).

Estimates of Dredge Efficiency

During transplant operations, the suction dredge covered an area of 1.8 and 2.6 hectares to extract 1,100 and 1,500 bushels of

material, respectively. An experimental plot was 10.63 hectares, so the suction dredge covered an area equivalent to 17% to 24% of the total plot area. Catch efficiency for live oysters (Eq. 1) varied from 19% to 58% (Table 1).

By comparison, the dry dredge covered an area of 4.2 and 6.5 hectares to extract 450 and 600 bushels of material, respectively, an area equivalent to 40% to 61% of the total plot area (Fig. 2). Catch efficiency was much lower than the suction dredge, about 5% (Table 1), a value similar to other reported catch efficiencies for traditional oyster dredges used in commercial mode (Banta et al. 2003).

In comparison, dredge efficiency of the *F/V Howard W. Sockwell* during sampling averaged much higher, 15% to 19%, than the 5% dredge efficiency for the equivalent gear during transplant operations, yielding an arithmetic-mean catchability coefficient of 6 to 7 (Table 4). We also present estimates of q as the least-square means or the average of least-square means for nonsignificant treatment-time pairs in Table 5 for later use in converting volumetric measures (bushel^{-1}) from dredge hauls to quantitative areal measures (m^{-2}).

We used the estimated dredge efficiency for the *F/V Howard W. Sockwell* sampling in survey mode to monitor bottom consolidation, presuming that a decrease in bottom consolidation would

TABLE 2.

The number of oysters per bushel per 1-cm size class in the dredge hopper, on the deck after passing through the culling machine, and on the bottom prior to dredging for each Cohansey plot worked by the dry-dredge boats. The concentration factor is the ratio between the number on-deck and in-hopper or the number on-deck and on-bottom for each 1-cm size class after standardization of each sample to 100 animals per bushel. Bottom estimates were obtained from diver samples taken prior to transplant. The bottom-to-deck concentration factor given in parentheses without standardization includes the change in abundance within the size class as well as the proportional shift in size frequency.

Treatment Plot (1-Cm Size Class)	Number per Bushel (In-Hopper)	Number per Bushel (On-Deck)	Hopper-to-Deck Concentration Factor	Number per Bushel (On-Bottom)	Bottom-to-Deck Concentration Factor	Treatment Plot (1-Cm Size Class)	Number per Bushel (In-Hopper)	Number per Bushel (On-Deck)	Hopper-to-Deck Concentration Factor	Number per Bushel (On-Bottom)	Bottom-to-Deck Concentration Factor
Dry-Dredge Plot 23						Dry-Dredge Plot 33					
1	0.0	0.0	0.0	0.0	—	1	0.0	0.0	0.0	0.0	—
2	4.8	5.0	0.8	0.0	∞	2	1.8	2.5	1.1	0.0	∞
3	29.2	33.6	0.9	8.9	(3.8) 1.4	3	14.8	17.0	0.9	7.2	(2.4) 1.0
4	78.8	79.4	0.8	29.0	(2.7) 1.0	4	49.6	55.4	0.8	22.1	(2.5) 1.0
5	92.1	103.6	0.9	44.3	(2.3) 0.9	5	88.1	94.0	0.8	42.0	(2.2) 0.9
6	72.2	90.3	1.0	38.9	(2.3) 0.9	6	81.6	115.3	1.1	43.6	(2.6) 1.1
7	31.2	56.8	1.5	20.0	(2.9) 1.1	7	46.4	79.7	1.3	25.5	(3.1) 1.3
8	16.0	28.4	1.4	10.1	(2.8) 1.1	8	18.1	30.5	1.3	14.8	(2.2) 0.9
9	3.8	8.2	1.7	2.6	(3.2) 1.2	9	8.2	11.5	1.1	7.7	(1.5) 0.6
10	0.8	3.5	3.4	1.5	(2.3) 0.9	10	4.0	2.9	0.6	2.6	(1.1) 0.4
11	0.2	0.9	3.9	0.0	∞	11	0.2	1.2	5.6	0.9	(1.3) 0.5
Σ ¹¹	329.1	409.7	—	155.3	(2.64) —	Σ ¹¹	312.8	410.0	—	166.4	(2.46) —

result in increased sampling efficiency of the dredge. Dredge efficiency of the *F/V Howard W. Sockwell* varied significantly between sampling times ($P < 0.0001$, Table 6, Fig. 6). Whether the plot was a control plot, an experimental plot impacted by the dry dredge, or an experimental plot impacted by the suction dredge did not significantly influence dredge efficiency; however, the interaction term between treatment and time was significant ($P = 0.0017$) because the time-history varied for each treatment (Table 6, Fig. 6). Prior to transplant, dredge efficiency was lowest on the plots to be dry-dredged, but dredge efficiency varied only between 7% and 14% among both treatment and control groups. After transplant, dredge efficiency rose significantly in the two treatments to 32% and 39% for the dry dredge and suction dredge, respectively, whereas dredge efficiency on the control plot remained low at 17% (Fig. 6). Ten months later, dredge efficiency had fallen to 20% on the suction dredge plots, remained high on the dry-dredge plots (34%), and risen on the control plots (35%) (Fig. 6). Thus, dredge efficiency was lowest in all cases prior to transplant, then rose rapidly shortly after transplant in the two treatments, but not in the control plot. Dredge efficiencies were higher 10 months later in all plots, having risen to treatment levels in the controls by that time. Because we do not know the extent of possible industry dredging during the 10 months after transplant, the upward trend in the controls cannot be broadly interpreted. However, prior dredging by dry dredge or suction dredge increased sampling efficiency of the *F/V Howard W. Sockwell* shortly after transplant.

Oyster size also significantly influenced dredge efficiency ($P = 0.025$). Dredge efficiency was lowest for smaller oysters <63.5 mm (17%) and higher for the rest (30%) (Tables 4, 6). Although less often significant, this trend was true within treatments for all time comparisons and within times for all but one treatment comparison, the exception being just prior to transplant.

As a consequence of the significant time \times treatment interaction, we also examined the change in dredge efficiency within

treatment over time using, as a metric, the magnitude of change in dredge efficiency between sampling periods (bottom set of analyses, Table 6). Dredge efficiency changed little in the control plots over the period of transplant, but changed significantly in plots subjected to dry or suction dredging over the same period ($\Delta 25\%$ in both treatments). The degree of change in dredge efficiency in dredged plots was significantly higher than in the control (LS means test, versus dry dredge, $P = 0.0023$; vs. suction dredge $P = 0.011$). The change in dredge efficiency was much higher in market-size animals ($\Delta 32\%$) than submarket-size ($\Delta 18\%$) and significantly higher than smaller ($\Delta 7\%$) animals. The difference between the dredge efficiency shortly after transplant and 10 months later was significant, but now the only change of consequence was the change in dredge efficiency observed in the control plots ($\Delta 17\%$) in comparison to the change observed in the two treatment groups (Table 6).

Before-treatment Similarity in Dredge and Control Plots

Cohansey—Dredge Boat

Three random 1-min dredge tows were taken per plot prior to transplant on May 9, 2002. Significant differences were not observed between plots in the number of oysters, boxes, or spat, or the amount of cultch per bushel ($\alpha = 0.05$).

The cross-sectional area of the largest clumps averaged larger for the dredge samples than the diver samples (Table 7, 8), probably because the bushel sample obtained by the dredge was much larger than the volume sampled by a diver. Further analysis showed that average clump size of the 12 largest clumps collected by the divers much more closely approximated the maximum average clump size observed in the dredge samples, supporting this supposition. Clump cross-sectional area varied significantly among plots ($P < 0.0001$). An *a posteriori* test showed a complex array of relationships generally separating plots 23 and 24 with lower clump cross-sectional areas from plots 33 and 34. The num-

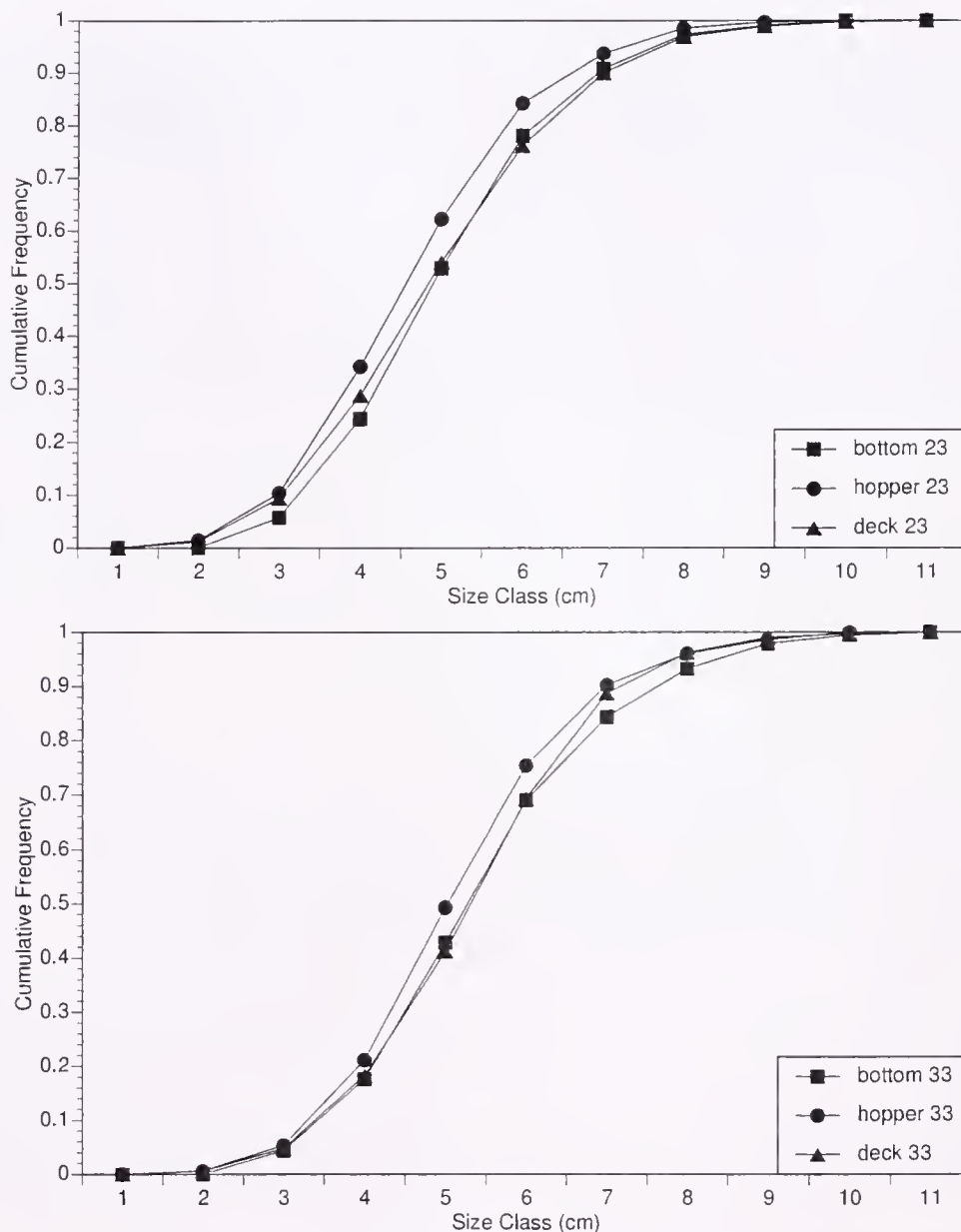


Figure 4. Cumulative size frequencies comparing the size structure of live oysters on the bottom, caught by the traditional oyster dredge (hopper), and passed through the culling machine to be transplanted downbay (deck). Numbers 23 and 33 refer to the grid plot numbers used for the experiment.

ber of oysters per clump also varied significantly among plots ($P < 0.0001$). Control plot 34B had clumps with significantly more live oysters per clump than any other. The same was not true for boxes.

Of the size-frequency metrics, only the 75th percentile of size varied significantly between plots ($P = 0.0055$), being significantly lower in plot 23 than plot 32. Thus, the largest oysters were larger on some plots than on others. The same was not true for boxes.

Cohansey—Diver Samples

Five random diver transects were sampled prior to transplant in each plot. The plots differed significantly in most measures prior to transplant, primarily because the volume of material per 0.25-m^2 collected on plot 32 destined for suction dredging was much

lower than the remainder and because proportionately more material collected from this plot was shell rather than live oysters, boxes, or debris. The trend carried through to the volume of culch ($P < 0.0001$). The two suction-dredge plots, 24 and 32, had the lowest culch coverage and the two dry-dredge plots, 23 and 33, had the highest (Table 9). The number of oysters per 0.25 m^2 also differed significantly between plots prior to transplant ($P < 0.0001$), as did the number of boxes ($P < 0.0001$). The control plot averaged higher than the others; plot 32 averaged considerably lower. Average clump cross-sectional area varied significantly between plots ($P < 0.0001$), being significantly smaller in plot 32. Not surprisingly, the number of oysters and boxes per clump also differed significantly among some plots ($P < 0.001$). Spat per 0.25 m^2 were significantly more common on the control plot ($P < 0.0001$) (Table 9); however the number of spat per clump did not

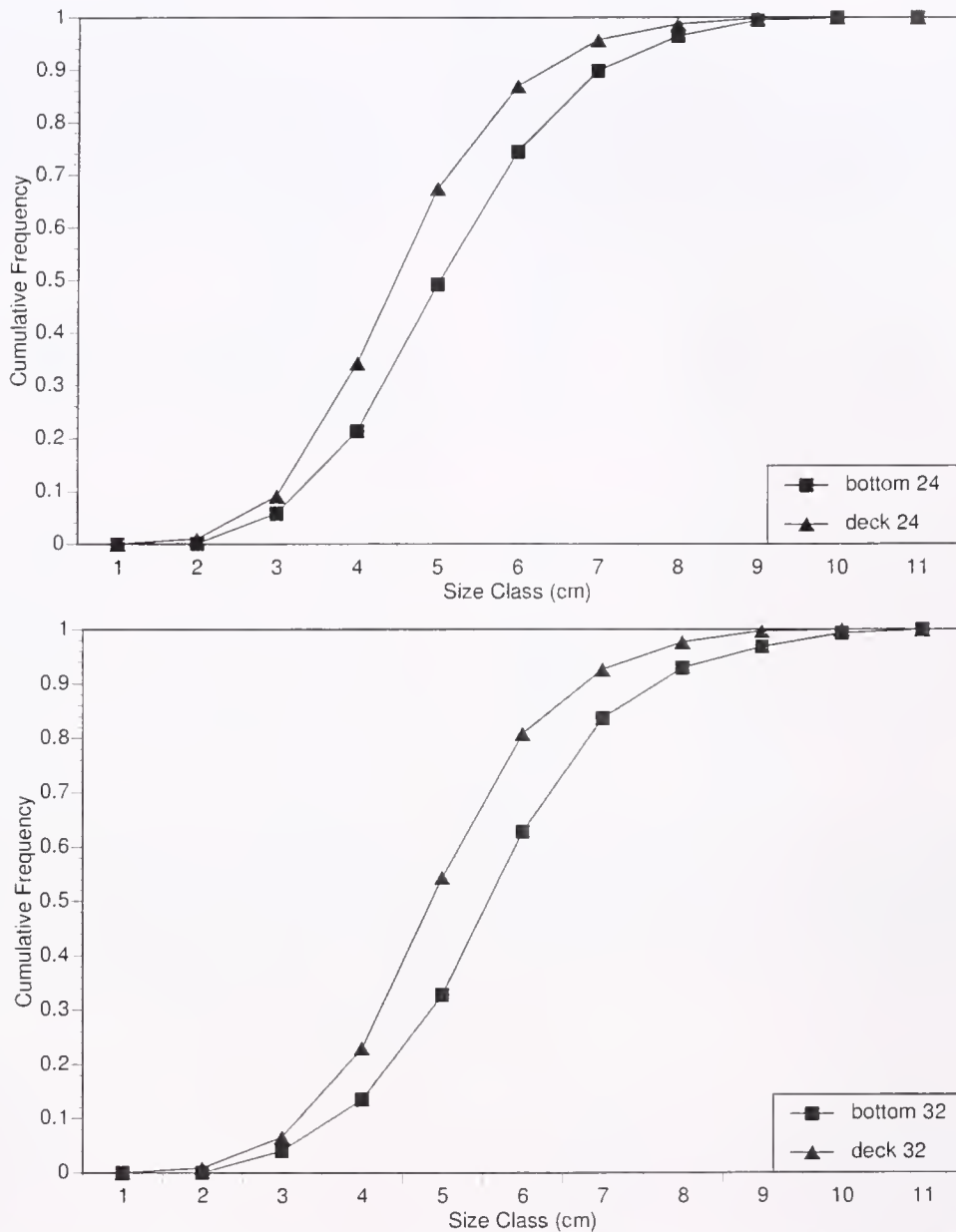


Figure 5. Cumulative size frequencies comparing the size structure of live oysters on the bottom and caught by the suction dredge to be transplanted downbay (deck). Numbers 24 and 32 refer to the grid plot numbers used for the experiment.

differ significantly between plots prior to transplant. Neither live oyster nor box size-frequency distribution differed significantly between plots prior to transplant. Finally, the average minimum number of clumps, defined as the 10 largest clumps or the total number of clumps if less than 10, differed significantly between plots prior to transplant ($P < 0.0001$) because plot 32 averaged fewer clumps than the remainder.

Overall, between-plot differences were more noticeable from the diver samples than the dredge-boat samples that integrated a much larger area of the bottom, but, in both cases, the size-frequency distribution was uniform, whereas oyster abundance and "clumpiness" differed. As a consequence, further analyses focused on time \times treatment interactions because between-treatment differences must be presumed to have originated from these prior differences in plot status.

Fate of Transplant Plots

Population Level Impact of Dredging—Near-term

Cohansey—Dredge Samples We compared treatment plots prior to and within 1 month after transplant. Treatments plots differed significantly (Tables 7, 10) only for the number of oysters per bushel. Very likely, this difference originated as plot differences prior to transplant. The time \times treatment interaction term was significant only for the number of oysters per clump (Table 10) which rose more in the plots subjected to the suction dredge (Table 7) than in the plots subjected to the dry dredge. A similar rise in the controls suggests that this result could be a sampling artifact. Similar results were obtained after a correction for dredge efficiency to quantify abundance (Table 5). The abundance of oysters and boxes

TABLE 3.

The number of oysters per bushel per 1-cm size class on the deck and on the bottom for each Cohansey plot worked by the suction-dredge boat. The concentration factor is the ratio between the number on-deck and on-bottom for each 1-cm size class after standardization of each sample to 100 animals per bushel. Bottom estimates were obtained from diver samples taken prior to transplant, except for plot 32. Divers collected an aberrantly low number of oysters prior to transplant on this plot. Accordingly, diver samples taken shortly after transplant were used. Numbers per bushel, thus, may be underestimated and concentration factors overestimated for this plot. The bottom-to-deck concentration factor given in parentheses without standardization includes the change in abundance within the size class as well as the proportional shift in size frequency.

Treatment Plot (1-Cm Size Class)	Number per Bushel (On-Deck)	Number per Bushel (On-Bottom)	Bottom-to-Deck Concentration Factor	Treatment Plot (1-Cm Size Class)	Number per Bushel (On-Deck)	Number per Bushel (On-Bottom)	Bottom-to-Deck Concentration Factor
Suction-Dredge Plot 24				Suction-Dredge Plot 32			
1	0.0	0.0	—	1	0.0	0.0	—
2	2.2	0.2	(11.0) 9.5	2	3.1	0.0	∞
3	18.4	11.2	(1.6) 1.4	3	20.7	5.6	(3.7) 1.4
4	57.0	30.4	(1.9) 1.6	4	59.7	13.2	(4.5) 1.7
5	75.4	54.7	(1.4) 1.2	5	113.8	26.8	(4.2) 1.6
6	44.4	49.7	(0.9) 0.8	6	96.0	41.7	(2.3) 0.9
7	19.9	30.1	(0.7) 0.6	7	42.9	29.0	(1.5) 0.6
8	6.7	12.9	(0.5) 0.4	8	18.4	12.9	(1.4) 0.5
9	2.5	5.8	(0.4) 0.3	9	7.4	5.4	(1.4) 0.5
10	0.5	1.0	(0.5) 0.4	10	1.0	3.4	(0.3) 0.1
11	0.0	0.2	(0.0) 0.0	11	0.0	1.0	(0.0) 0.0
Σ _{i=1} ¹¹	227.0	196.2	(1.16) —	Σ _{i=1} ¹¹	363.0	139.0	(2.61) —

did not vary significantly over treatment or time (# m⁻², Table 10), and the time × treatment interaction term was not significant.

Condition index varied significantly over time, as might be anticipated by the seasonal cycle of condition index in oysters (Hopkins et al. 1954, Haven 1962, Versar Inc. 2002), but the interaction term was not significant (Tables 10, 11). The size-

frequency distributions did not change significantly by any metric (Table 12). Because transplantation focuses on submarket and market-size animals, we also examined the proportion of small animals (<63.5 mm), submarket-size animals (63.5–<76.2 mm), and market-size animals (>76.2 mm). These proportions differed significantly over time, but the time × treatment interaction term was not significant (Table 10).

TABLE 4.

Catchability coefficients *q*, calculated as the reciprocal of dredge efficiency [equation (1)], for the sampling vessel, the *F/V Howard W. Sockwell*, using an oyster dredge in survey mode (Powell et al., 2002). Oyster size classes are market-size oysters, submarket-size oysters expected to grow to market size within one year (HSRL, 2003), and smaller oysters. Before, the initial sampling prior to transplantation; After, the sampling shortly after transplantation; Later, the sampling 10 months after transplantation.

Treatment	Time	Oysters <63.5 mm Mean <i>q</i>	Oysters 63.5–<76.2 mm Mean <i>q</i>	Oysters ≥76 mm Mean <i>q</i>
Control	Before	10.51	8.14	9.54
	After	6.65	4.96	6.16
	Later	3.18	2.78	2.72
Dry Dredge	Before	14.99	13.55	17.06
	After	5.05	2.95	3.62
	Later	4.70	2.38	2.59
Suction Dredge	Before	6.56	6.66	9.33
	After	5.51	3.08	1.77
	Later	6.42	5.49	3.95
<i>Average ± Std</i>				
Control		6.03 ± 3.08	4.73 ± 2.22	5.46 ± 3.02
Dry Dredge		8.25 ± 5.47	6.29 ± 5.66	7.76 ± 7.35
Suction Dredge		6.00 ± 1.09	4.58 ± 1.85	4.21 ± 3.60
Grand Average ± Std		6.91 ± 3.86	5.31 ± 3.77	6.04 ± 5.20

Cohansey—Diver Samples Comparison can be made to the same analyses using diver samples. Neither time nor treatment exerted a significant main effect; however, the treatment × time interaction term was significant (Table 10) because a decrease in oyster abundance occurred, particularly in the dry-dredge plots relative to the controls (Table 8). The same was not true for boxes (Tables 8, 10). The treatment × time interaction term was significant for maximum clump cross-sectional area that declined on plots subjected to the dry dredge, but did not vary much in the control or suction-dredge plots (Tables 8, 10).

The number of oysters and boxes per clump did not vary significantly between treatments or over time (Table 10). The treatment × time interaction term was highly significant in both cases, however (Table 10). The number of live oysters and boxes tended to decline in plots subjected to dry dredging relative to the controls (Table 8). The number of spat per clump did not change significantly over time, nor did the average minimum number of clumps per m² (Tables 8, 10). The size-frequency distribution of live oysters and boxes did not vary over time by any of the five metrics used.

Population Level Impact of Dredging—Long-term

Cohansey—Dredge Samples We compared the condition of plots over a 10-month period after transplant. Not surprisingly, the number of oysters and boxes differed significantly over time, as

TABLE 5.

Quantitative estimates of oyster abundance for each treatment and date of sampling using the least-square means estimates of q obtained from statistical analysis of differences in catchability versus treatment (control, dry-dredge, suction-dredge) and date of sampling in Table 6. Average values of q were taken in cases where significant differences were not detected between treatments.

Treatment	Oysters m ⁻² May 9, 2002	Oysters m ⁻² July 15, 2002	Oysters m ⁻² November 5, 2002	Oysters m ⁻² May 6, 2003
<i>Dredge Samples</i>				
Control	43.8	102.2	16.0	25.2
Dry Dredge	46.1	26.3	10.3	16.6
Suction Dredge	25.7	27.3	21.6	18.4
<i>Average LSmeans Dredge Efficiency</i>				
Control	9.29	5.72	—	2.86
Dry Dredge	14.93	3.01	—	3.01
Suction Dredge	7.32	3.40	—	3.40

did the number of spat (Tables 7, 13). However, in most cases, these differences originated in the differences that originally existed between plots. Similar results were obtained after a correction for dredge efficiency to quantify oyster abundance (# m⁻²). The abundance of oysters and boxes did not vary significantly over treatment, but the abundance of oysters did change over time (Tables 7, 13). The time \times treatment interaction term was not significant. The time \times treatment interaction term was significant

for the number of oysters per clump and clump cross-sectional area (Tables 7, 13). Over 10 months, the number of oysters per clump declined in all treatments, but lesser declines occurred in the plots subjected to dry dredging (Table 7). Average maximum clump cross-sectional area declined, but only in the control plots.

Condition index varied significantly over time in accordance with the seasonal cycle, but the interaction term was not significant (Tables 11, 13). The size-frequency distribution changed signifi-

TABLE 6.

Results of ANOVA analysis of dredge efficiency using the comparison between the sampling dredge in survey mode operated by the F/V *Howard W. Sockwell* and the quantitative diver samples. Data and remaining details appear in Table 4. Treatment comprises the control plots, plots subjected to the dry dredge, and plots subjected to the suction dredge. Time represents the three sampling times, prior to transplant, shortly after transplant, and 10 months after transplant. After-Before, the difference between samples taken just before and just after transplant. Ten Months Later-After, the difference between samples taken just after transplant and about 10 months later. Size class represents market-size oysters, ≥ 76.2 mm, submarket-size oysters, 63.5 mm to < 76.2 mm, and smaller oysters, < 63.5 mm. —, not significant at $\alpha = 0.05$.

Time			$P < 0.0001$
Size Class			$P = 0.025$
Time \times Size Class			—
Treatment			—
Time \times Treatment			$P = 0.0017$
Size Class \times Treatment			—
Time \times Size Class \times Treatment			—
ANOVA by Treatment		ANOVA by Time	
Control		Before Transplant	
Time	$P = 0.0005$	Treatment	$P = 0.024$
Size Class	—	Size Class	—
Time \times Size Class	—	Treatment \times Size Class	—
Dry Dredge		Shortly After Transplant	
Time	$P = 0.001$	Treatment	$P = 0.033$
Size Class	—	Size Class	—
Time \times Size Class	—	Treatment \times Size Class	—
Suction Dredge		Ten Months Post-Transplant	
Time	$P = 0.032$	Treatment	$P = 0.018$
Size Class	—	Size Class	$P = 0.041$
Time \times Size Class	—	Treatment \times Size Class	—
ANOVA by Time Difference			
After-Before Transplant			
Treatment			$P = 0.0057$
Size Class			$P = 0.02$
Treatment \times Size Class			—
Ten Months Later-After Transplant			
Treatment			$P = 0.0065$
Size Class			—
Treatment \times Size Class			—

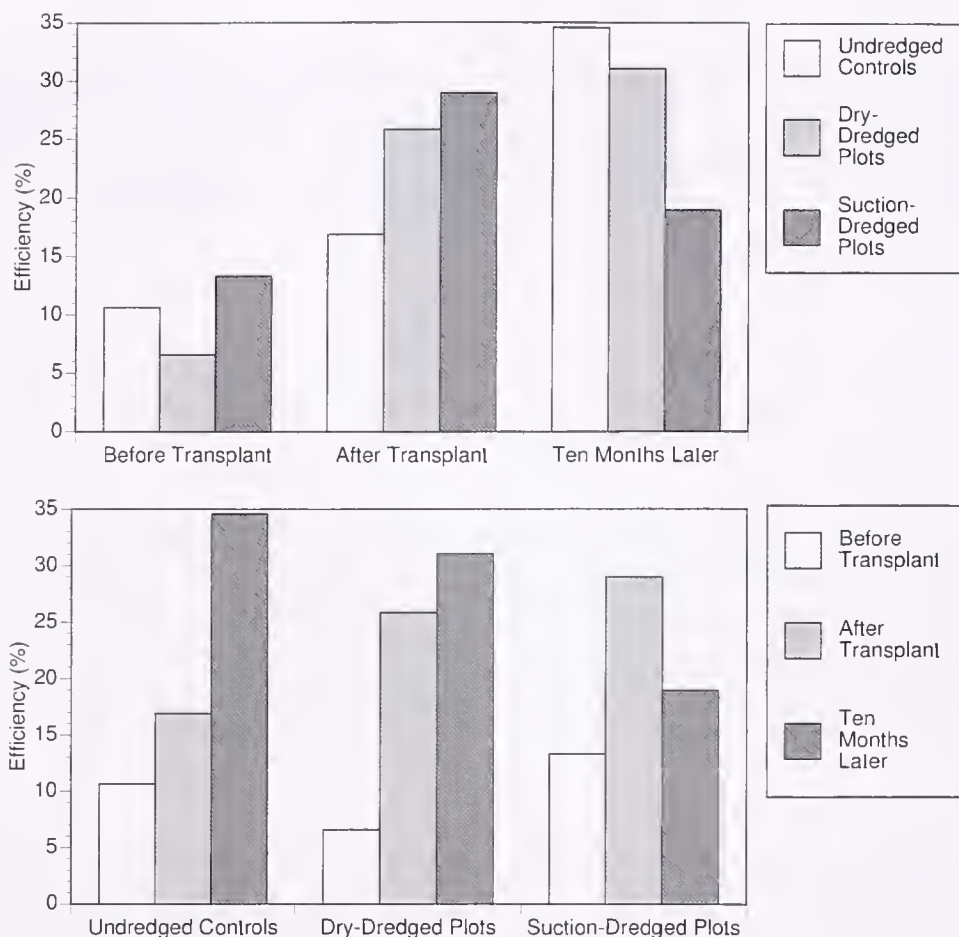


Figure 6. Comparison of dredge efficiency as a percentage for each treatment and time period.

TABLE 7.

Status of plots at each sampling time as evaluated by dredge samples standardized to the bushel.

Treatment	Oysters (bushel ⁻¹)	Boxes (bushel ⁻¹)	Maximum Clump Cross- Sectional Area (cm ²)	Oysters (clump ⁻¹)	Boxes (clump ⁻¹)
<i>May 9, 2002</i>					
Control	339.7	23.0	72.6	3.67	0.13
Dry Dredge	250.1	24.6	58.1	2.02	0.22
Suction Dredge	173.6	18.6	54.4	1.55	0.27
<i>July 15, 2002</i>					
Control	427.7	34.5	80.4	4.14	0.36
Dry Dredge	242.4	30.1	71.8	2.67	0.36
Suction Dredge	260.0	30.6	71.5	3.20	0.35
<i>November 5, 2002</i>					
Control	174.9	40.2	68.4	2.17	0.35
Dry Dredge	150.0	35.2	59.2	1.39	0.37
Suction Dredge	218.0	49.1	91.4	2.57	0.48
<i>May 6, 2003</i>					
Control	190.6	59.4	71.9	2.24	0.67
Dry Dredge	150.6	46.0	69.1	1.47	0.40
Suction Dredge	126.8	45.3	74.8	1.20	0.47

TABLE 8.
Status of plots at each sampling time as evaluated by quantitative diver samples.

Treatment	Oysters (0.25 m ⁻²)	Boxes (0.25 m ⁻²)	Maximum Clump Cross- Sectional Area (cm ²)	Oysters (clump ⁻¹)	Boxes (clump ⁻¹)	Spat (clump ⁻¹)	Average Minimum Clump Number
<i>June 10, 2002</i>							
Control	26.1	7.4	46.4	1.45	0.34	0.05	7.0
Dry Dredge	19.8	5.1	46.3	1.20	0.25	0.03	6.5
Suction Dredge	8.7	2.0	30.6	0.68	0.14	0.02	5.3
<i>June 24, 2002</i>							
Control	28.0	6.0	43.1	1.48	0.29	0.07	7.5
Dry Dredge	10.4	3.2	34.0	0.80	0.21	0.02	6.2
Suction Dredge	8.3	2.7	36.1	0.82	0.22	0.03	5.3
<i>April 28, 2003</i>							
Control	15.1	7.5	45.6	1.03	0.44	0.01	7.0
Dry Dredge	7.2	4.1	31.8	0.50	0.21	0.00	6.6
Suction Dredge	10.1	6.4	38.6	0.70	0.39	0.00	5.9

cantly over time (Table 13). Mean and median size rose, as did the 75th and 25th percentile (Table 12). The interquartile range changed little. Thus, growth occurred more or less equivalently on all plots. The time \times treatment interaction term was significant only for the interquartile range, and then barely so (Table 13). Because transplantation focuses on submarket and market-size animals, we also examined the proportion of small, submarket, and market-size animals. At this coarser level of resolution, the size-frequency distribution did not change significantly (Table 13).

Cohansey—Diver Samples Ten months after transplant, the number of live oysters had declined significantly (Table 13). The treatment \times time interaction term was highly significant (Table 13). Declines were largest in the controls; abundance was relatively unchanged in the suction-dredge plots (Table 8). The number of boxes did not vary much over time, nor was the interaction with treatment significant (Table 13).

Maximum clump cross-sectional area varied significantly over time. The treatment \times time interaction was highly significant (Table 13). The number of live oysters, boxes, and spat per clump all varied significantly over time and the interaction term with treatment in all cases was significant (Table 13). The number of

boxes per clump increased substantially in the control and suction-dredge plots in comparison to the dry-dredge plots (Table 8). The number of live oysters per clump declined, particularly on plots that had been impacted by the dry dredge and in the controls. The number of spat fell, particularly in control plots (Table 9); however, few spat were recorded in any plot. The minimum average number of clumps changed little (Table 13).

The size-frequency distributions changed significantly over time (median size, $P = 0.0028$; mean size $P = 0.0072$; 75th percentile, $P = 0.018$; 25th percentile, $P = 0.055$) because oyster size increased during the 10-months elapsed time in all plots and only a limited number of spat grew into the smaller size classes (Table 14). Size frequency was not significantly impacted by treatment.

Transplant Effects on Habitat Complexity

Suction dredges and dry dredges operate differently and might, thereby, differentially influence the small-scale structure of the bottom. We evaluated this possibility in a number of ways previously discussed, including the measurement of maximum clump cross-sectional area, the minimum average number of clumps

TABLE 9.
Average volumetric composition and number of spat per 0.25 m² from diver samples.

Treatment	Sample Volume (L)	Live Oyster Volume (L)	Box Volume (L)	Cultch Volume (L)	Debris Volume (L)	Number of Spat (0.25 m ⁻²)
<i>June 10, 2002</i>						
Control	4.32	2.25	0.39	1.56	0.10	0.68
Dry Dredge	4.08	1.43	0.30	2.21	0.13	0.31
Suction Dredge	1.98	0.63	0.14	1.14	0.07	0.13
<i>June 24, 2002</i>						
Control	5.16	3.02	0.31	1.73	0.09	0.67
Dry Dredge	2.70	0.90	0.21	1.51	0.06	0.24
Suction Dredge	2.16	0.77	0.18	1.13	0.05	0.19
<i>April 28, 2003</i>						
Control	3.36	1.49	0.40	1.40	0.07	0.17
Dry Dredge	3.06	0.77	0.35	1.85	0.08	0.07
Suction Dredge	2.87	0.79	0.41	1.57	0.09	0.06

TABLE 10.

Results of repeated measures ANOVA comparing the results of diver sampling and dredge sampling of the Cohansey plots prior to transplant and less than one month after transplant. —, not significant at $\alpha = 0.05$. The test for condition index used meat dry weight as the dependent variable and shell area ($A = \text{length} \times \text{height}$) as a covariate. The tests for size-frequency proportions used the number of animals >76.2 mm per bushel as the covariate and the number of submarket and smaller oysters per bushel as the dependent variables.

Treatment	Oysters (0.25 m ⁻²)	Boxes (0.25 m ⁻²)	Maximum Clump Cross- Sectional Area (cm ²)	Oysters (clump ⁻¹)	Boxes (clump ⁻¹)	Spat (clump ⁻¹)	Average Minimum Clump Number
<i>Diver Samples</i>							
Treatment	—	—	—	—	—	$P = 0.019$	—
Plot (Treatment)	$P = 0.0022$	$P = 0.0006$	$P < 0.0001$	$P < 0.0001$	$P = 0.0005$	—	$P = 0.012$
Time	—	—	—	—	—	—	—
Treatment \times Time	$P = 0.0048$	—	$P < 0.0001$	$P < 0.0001$	$P = 0.0037$	—	—
Treatment	Oysters (bushel ⁻¹)	Boxes (bushel ⁻¹)	Maximum Clump Cross- Sectional Area (cm ²)	Oysters (clump ⁻¹)	Boxes (clump ⁻¹)	Spat bushel ⁻¹	
<i>Dredge Samples</i>							
Treatment	$P = 0.0024$	—	—	—	—	—	—
Plot (Treatment)	—	—	$P < 0.0001$	$P = 0.0016$	—	—	—
Time	—	—	$P < 0.0001$	$P < 0.0001$	$P = 0.021$	—	—
Treatment \times Time	—	—	—	$P = 0.012$	—	—	—
Treatment	Oysters (# m ⁻²)	Boxes (# m ⁻²)	Condition Index	Proportion <63.5 mm	Proportion 63.5–<76.2 mm		
<i>Dredge Samples</i>							
Treatment	—	—	—	—	—	—	—
Plot (Treatment)	—	—	—	$P < 0.0001$	—	—	—
Time	—	—	$P < 0.0001$	$P < 0.0001$	—	—	—
Treatment \times Time	—	—	—	—	—	—	—

0.25-m⁻², and the number of live oysters and boxes per clump. We also examined complexity through analysis of the variability in the distribution of oysters and boxes using as descriptive metrics the variance between samples, the range of sample values, and the skewness and kurtosis of the sample values.

We first examined the variability between the 12 samples taken within each diver transect. The metrics were not significant for oysters or boxes, with the single exception of skewness among oyster values ($P = 0.044$). Nothing suggests that the distribution of sample values among samples within diver transects differed between treatments or over time. We also examined the variability between the five diver transects taken within each plot using the same four metrics. Once again, no significant differences were found in any of the four metrics.

Finally, we examined the proportional contribution of oysters, boxes, cultch, and debris to the volume collected by diver, on the

premise that removal of material during transplant should have influenced composition. Treatments never differed significantly in these volumetric proportions (Table 15). Time did significantly influence sample proportions, particularly live oysters and boxes (Table 15). Live oyster volume declined over time and box volume increased in most treatments. The time \times treatment interaction terms were significant in all cases except cultch volume (Table 15). For live oysters, a change in proportional contribution occurred because the volume of live oysters declined noticeably on control plots relative to either treatment over time (Table 9).

The expectation that cultch volume should be affected in some way led to a further examination of the variability in cultch volume among samples, as measured by the variance. The variance in cultch volume between samples on a diver transect was significantly higher 10 months after transplant than at earlier times, but the time \times treatment interaction term was not significant (Table 15).

TABLE 11.

Condition index [g dry wt (cm² shell area)⁻¹] for each plot at each sampling date.

Treatment	Condition Index May 9, 2002	Condition Index July 15, 2002	Condition Index November 5, 2002	Condition Index May 6, 2003
<i>Dredge Samples</i>				
Control	0.0287	0.0342	0.0188	0.0247
Dry Dredge	0.0282	0.0344	0.0180	0.0233
Suction Dredge	0.0273	0.0339	0.0187	0.0226

TABLE 12.

Metrics of the size-frequency distribution from dredge samples taken from the Cohansey plots.

Treatment	75 th			25 th
	Mean Size (mm)	Percentile Size (mm)	Median Size (mm)	Percentile Size (mm)
<i>May 9, 2002</i>				
Control	54.3	63.7	53.8	44.5
Dry Dredge	51.7	59.7	50.5	41.9
Suction Dredge	54.9	63.1	54.5	45.7
<i>July 15, 2002</i>				
Control	55.0	64.6	54.6	44.8
Dry Dredge	55.1	63.7	54.9	46.0
Suction Dredge	57.7	66.8	57.2	48.7
<i>November 5, 2002</i>				
Control	58.7	68.3	57.9	48.7
Dry Dredge	57.9	66.3	56.9	48.7
Suction Dredge	59.4	68.5	58.7	49.2
<i>May 6, 2003</i>				
Control	58.9	67.0	57.7	50.2
Dry Dredge	58.5	67.6	58.1	48.8
Suction Dredge	60.4	70.2	59.6	50.3

Perhaps an effect on complexity can only be resolved by comparing bed status just before and just after transplant. We repeated each of the previous analyses, looking specifically at this time interval. No significant trends were revealed.

Fate of Transplants on New Beds

The number of oysters per bushel was not significantly elevated after transplant on either New Beds plot. Clump cross-sectional area declined significantly ($P < 0.0001$) from 71.4 cm² to 47.4 cm², however, and the number of oysters per clump increased from 0.7 to 1.8 ($P < 0.0001$). The size-frequency of live oysters did not change significantly, nor did the proportions of small, submarket, and market-size animals. However, condition index rose significantly in both plots, from the prior value of 0.049 g dry wt (cm² shell area)⁻¹ to 0.068 and 0.052 g dry wt (cm² shell area)⁻¹ for the dry-dredge and suction dredge plots, respectively ($P = 0.0007$). Thus, evidence that the oysters transplanted to New Beds changed population structure therein was revealed by a number of metrics.

The New Beds plots were followed for about 10 months after transplant to determine if the fate of the transplanted animals diverged between animals transplanted by dry dredge and by suction dredge. Few significant differences were observed between treatments or over time. Only a single time \times treatment interaction term was significant, for the number of oysters per clump ($P = 0.0024$). The number of live oysters per clump declined over the 10 month from 1.6 and 2.0 for the dry-dredge and suction-dredge transplants, respectively, to 0.5 and 0.7 in May 2003 ($P < 0.0001$). The number of boxes per clump simultaneously rose significantly from 0.13 and 0.13 to 0.60 and 0.50, respectively ($P = 0.0012$). The mortality occurred during a Dermo epizootic that recorded the highest overall oyster mortality on record for the 1989 to 2002 period (HSRL 2003). *Perkinsus marinus* prevalence and infection intensity were not significantly different between the transplant plots and the control plot in May 2003.

Condition index changed significantly after transplant, following the expected seasonal cycle, with highest values recorded in July 2002 and lowest values recorded the following November

($P < 0.0001$). During this 10-month period, the dry-dredge plots averaged significantly higher in condition index than the suction-dredge plots on each sampling occasion ($P < 0.0001$) (July, 2002: 0.068 vs. 0.052 g dry wt (cm² shell area)⁻¹; November, 2002: 0.035 vs. 0.026 g dry wt (cm² shell area)⁻¹; May, 2003: 0.051 vs. 0.039 g dry wt (cm² shell area)⁻¹). The time \times treatment interaction term, however, was not significant, suggesting that the differential between plots was established at the time of transplant.

DISCUSSION

Perspective

Oysters on the Delaware Bay nursery beds (Round Island to Cohansey beds, Fig. 1 in Banta et al. 2003) suffer relatively low annual mortality rates (Powell et al. 1997, Ford et al. 1999, Paraso et al. 1999), but do not routinely attain market quality due to small size and low condition index. Utilization of this resource requires downbay transplantation (Powell et al. 1997, Ford 1997, Kraeuter et al. 2003), whereupon submarket and market-size animals rapidly increase condition (Kraeuter et al., 2003) and relatively rapidly increase in size due to the increased food supply available at higher salinity (Powell et al. 1997, Versar Inc. 2002). Unfortunately, the higher salinity also brings with it markedly increased mortality rates from disease, principally Dermo caused by *Perkinsus marinus*, and, for small oysters, predation from crabs and oyster drills (Carriker 1955, Manzi 1970, Bisker & Castagna 1987). Thus, smaller oysters transplanted downbay requiring more than one growing season to reach market size will sustain considerable mortality prior to harvest.

The impact of oyster harvesting by tong or dredge has received much study (e.g., Marshall 1954, Lenihan & Micheli 2000, Mann 2000, Powell et al. 2001), but the specialized activity of transplanting has received much less. Oystering can significantly impact oyster populations and degrade oyster habitat (e.g., Rothschild et al. 1994, Peterson et al. 2000, Lenihan et al. 2001). Judging from the limited literature on transplanting, transplanting has not always been conducted sustainably (Moore 1911, Hargis & Haven 1988, Rothschild et al. 1994). Advantageous use of the oyster resource on the nursery beds of Delaware Bay has 3 requisites. (1) Large animals must be efficiently culled and moved downbay. These animals escape the increased predation rates and can be marketed quickly before disease mortality takes its toll. (2) The upbay nursery beds must be managed to achieve sustainable yield, in this case, sustainable transplant. (3) The transplant process must take place with limited long-term damage to the bed. Thus, transplant of shell should be limited as well as that of small live oysters.

The approach taken by New Jersey to ensure population stability on the nursery beds is to limit transplant to 10% of submarket + market-size abundance in any 1 year (HSRL 2003). This limits fishing mortality rate to a level generally considered sustainable (Rothschild et al. 1994, Klinck et al. 2001, Jordan et al. 2002). The transplant program also attempts to control the number of small oysters and the amount of shell transplanted downbay by limiting the fraction of the deck load that is cultch to $\leq 20\%$. But, the cost of this activity is high because oyster boats with culling machines, using traditional dry dredges, cannot move more than about 900 bushels per boat per day, typically at a cost of \$1.00 to \$2.00 per bushel. A suction dredge can move several times this quantity per day at a lower per-bushel cost. As a consequence, the suction dredge may be economically advantageous. However, use of the suction dredge must not compromise the requirements of sustainability, hence the motivation for this research program.

TABLE 13.

Results of repeated measures ANOVA comparing the results of dredge and diver sampling of the Cohansey plots within one month of transplant, after 10 months for diver samples, and after 4 and 10 months for dredge samples. —, not significant at $\alpha = 0.05$. ANCOVA tests as described in Table 9.

	Oysters (0.25 m ⁻²)	Boxes (0.25 m ⁻²)	Maximum Clump Cross- Sectional Area (cm ²)	Oysters (clump ⁻¹)	Boxes (clump ⁻¹)	Spat (clump ⁻¹)	Average Minimum Clump Number
<i>Diver Samples</i>							
Treatment	—	—	$P = 0.054$	—	—	—	—
Plot(Treatment)	$P < 0.0001$	$P = 0.0006$	$P < 0.0001$	$P < 0.0001$	$P = 0.034$	$P < 0.0001$	$P = 0.0005$
Time	$P = 0.0081$	—	$P = 0.029$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	—
Treatment \times Time	$P = 0.0024$	—	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P = 0.0033$	$P = 0.040$
	Oysters (bushel ⁻¹)	Boxes (bushel ⁻¹)	Maximum Clump Cross- Sectional Area (cm ²)	Oysters (clump ⁻¹)	Boxes (clump ⁻¹)	Spat bushel ⁻¹	
<i>Dredge Samples</i>							
Treatment	—	$P = 0.014$	—	—	—	—	—
Plot(Treatment)	—	—	$P < 0.0001$	$P < 0.0001$	—	—	—
Time	$P < 0.0001$	$P = 0.0063$	—	$P < 0.0001$	—	$P < 0.0001$	—
Treatment \times Time	—	—	$P < 0.0001$	$P < 0.0001$	—	—	—
	Oysters (# m ⁻²)	Boxes (# m ⁻²)	Condition Index	Proportion <63.5 mm	Proportion 63.5–76.2 mm		
<i>Dredge Samples</i>							
Treatment	—	—	—	—	$P = 0.016$	—	—
Plot(Treatment)	—	—	—	$P = 0.019$	—	—	—
Time	$P = 0.013$	—	$P < 0.0001$	—	—	—	—
Treatment \times Time	—	—	—	—	—	—	—
	Mean size	Median Size	25 th Percentile	75 th Percentile	Interquartile Range		
<i>Dredge Samples</i>							
Treatment	—	—	—	—	—	—	—
Plot(Treatment)	$P = 0.0002$	$P < 0.0001$	$P = 0.0004$	$P = 0.0018$	$P = 0.025$	—	—
Time	$P = 0.001$	$P = 0.0008$	$P = 0.0013$	$P = 0.029$	—	—	—
Treatment \times Time	—	—	—	—	$P = 0.049$	—	—

Operation of Dry and Suction Dredges

The traditional oyster dredge, a dry dredge, when towed in survey mode using short tows in which the dredge is retrieved without filling the bag, achieves dredge efficiencies on the order of 15% to >50% (Powell et al. 2002). Catch efficiency tends to be

much better for larger particles, including market-size oysters (Powell et al. 2002), as is true for most, if not all, types of shellfish dredges (e.g., Giguère & Brulotte 1994, Rudders et al. 2000, Volstad et al. 2000). Catch efficiency tends to be lowest for cultch, that is, single and fragmented valves without attached live oysters or

TABLE 14.

Metrics of the size-frequency distribution from diver sampling of the Cohansey plots after transplanting.

Treatment	Median Size (mm)	Mean Size (mm)	Interquartile Range (mm)	75 th Percentile Size (mm)	25 th Percentile Size (mm)
June 24, 2002					
Control	50.5	51.6	17.3	60.1	42.8
Dry Dredge	52.7	54.2	17.5	62.4	44.9
Suction Dredge	53.3	54.7	20.1	64.9	44.8
April 28, 2003					
Control	54.7	56.6	20.0	66.3	46.8
Dry Dredge	55.6	55.9	17.1	64.9	47.8
Suction Dredge	58.7	58.3	20.3	67.8	47.6

TABLE 15.

Results of repeated measures ANOVA comparing the results of diver sampling of the Cohansey plots within one month of transplant and after 10 months. —, not significant at $\alpha = 0.05$. All tests on volumetric fractions, except total volume used total volume as a covariate. The mean was used as a covariate in the test using the variance as a dependent variable.

	Sample Volume	Live Oyster Volume	Box Volume	Cultch Volume	Debris Volume	Spat Number (m ²)	Cultch Volume Variance
Treatment	—	—	—	—	—	—	—
Plot(Treatment)	$P = 0.0003$	$P = 0.0013$	$P = 0.0009$	$P < 0.0001$	$P < 0.0001$	—	—
Time	—	$P = 0.034$	$P = 0.0002$	$P = 0.02$	—	$P < 0.0001$	$P = 0.0073$
Treatment \times Time	$P = 0.012$	$P < 0.0001$	$P = 0.0043$	—	$P = 0.016$	$P = 0.03$	—

boxes (Powell et al. 2002). The survey dredge used for sampling in this study, onboard the *FIV Howard W. Sockwell*, behaved precisely as anticipated. Average dredge efficiency over all sampling periods was lower for the smaller size classes. Depending on particle size, dredge efficiency varied from 18% to 30%.

Under commercial operations, the dry dredge achieves a much lower dredge efficiency, typically below 5% (Banta et al. 2003). During commercial harvesting, oysters caught by the dredge are first passed through a culling machine, if one is installed, and then further hand-culled. The contrariety between survey and commercial operations arises from the fact that hand-culling onboard, required by marketing constraints, is much slower than the catching rate of the dredge. As a consequence, the dredge is left on the bottom longer than necessary, becomes overly full, and dredge efficiency necessarily then declines (Banta et al. 2003). Culling machines are used during transplant, but hand culling does not occur, and this speeds processing on deck. Nevertheless, for the culling machine to work properly, on-deck processing is still slow in comparison to the catching rate of the dredge. As a consequence, the dredge is left on the bottom longer than necessary and dredge efficiency necessarily then declines. Both dry-dredge boats used for transplanting in this study had dredge efficiencies of about 5.5%.

The propensity for allowing the dredge to remain on the bottom while deck culling proceeds, which leads to low dredge efficiency, has 2 unfortunate consequences. First, total swept area rises dramatically, about 100 bushels being loaded per hectare swept. Second, the tendency for the dredge to preferentially catch larger particles is negated: as the dredge fills, smaller particles are more likely to be retained. Presumably, larger particles can no longer easily fit into the bag. Catch efficiency rises for smaller particles including cultch and juvenile oysters, so material placed in the hopper for deck culling is enriched in precisely the less-desirable size classes targeted for culling and return to the bottom. Because the culling machine selects for the largest particles, the antithetical and unwanted effect of the overly-full dredge is largely counter-weighted. The final size-frequency distribution of the oysters on deck, integrating the two countervailing sorting processes, differs little from that present on the bottom. Clearly, the traditional oyster dredge as operated does not achieve the selectivity potential inherent in its design and desired in a sustainable transplant program. Despite the limitations however, the deck loads on the dry-dredge boats contained a factor of 2 to 3 more oysters per bushel than present on the bottom. So, cultch was selectively excluded, thereby protecting necessary substrate for the future larval settlement needed to replace oysters removed during the transplant process.

The suction dredge operated very differently. Catch efficiencies were high, varying between 19% and 58%. Total swept area is,

therefore, much lower for a given deck load. The suction dredge loaded about 600 bushels per hectare swept, about 6 times that achieved by the dry dredge. Efficiencies of 19% to 58% are in the same range as the catch efficiency of the dry dredge in survey mode.

The suction dredge has no onboard culling capability; consequently selection only occurs as material is removed from the bottom by the dredge. Nevertheless, the deck load averages 1.9 times more oysters per bushel than a quantitative sample of bottom material. However, suction dredging enriches the deck load in small particles, so that most of this increased abundance is small oysters. Presumably, some proportion of the larger animals escape capture because of their greater weight and concomitant larger sinking velocity or because they are more frequently affixed to the bed rather than lying loose on the bottom. Thus, surprisingly, both dredges as operated select similarly on the bottom, for small particles, the suction dredge being somewhat more successful in this undesirable trait. The amount of cultch retained differs dramatically, however. The deck load from the suction dredge was 62% cultch compared with only 27% for the dry dredge.

Effect of Dredging on Dredge Efficiency in Survey Mode

Dredge efficiency measurements are notoriously variable (e.g., Beukers-Stewart et al. 2001, Powell et al. 2002, NEFSC 2003). One typically ascribes this variability to the inherent variability of measurement and some controlling aspect of bottom condition. Powell et al. (2002) identified two regions in Delaware Bay that had consistently divergent dredge efficiencies, one with efficiencies averaging 65% for market-size animals and the other with efficiencies averaging 14% for market-size animals. Areas with characteristically high dredge efficiencies were on the downbay oyster beds used for commercial harvest and, thus, dredged frequently and intensively (Banta et al. 2003). Areas with characteristically low dredge efficiencies were upbay nursery beds in which dredging activity was limited (Banta et al. 2003). Powell et al. (2002) suggested that the difference in dredge efficiency could be ascribed to the degree of bottom consolidation that would vary with the frequency of dredging.

Both traditional dry and suction dredging break up the bottom. Sampling efficiency rose markedly after transplanting, from 6% beforehand to 28% afterwards for market-size oysters on the plots worked by the dry-dredge boat and from 11% beforehand to 56% afterwards for market-size oysters on the plots worked by the suction-dredge boat. At the same time, sampling efficiency rose from 10% to only 16% on the control plots. Anecdotal observations by oystermen suggest that bottom cohesion increases over the winter and then declines in the spring as the weather warms. Such a process would explain the small rise in dredge efficiency on the

control plots between May and July 2002. The more dramatic change on the dredged plots can only be ascribed to dredging breaking up the bed surface. The dry dredge does this mechanically as the dredge teeth are scraped over and forced to bite into the bottom. How the suction dredge operates to reduce bed cohesion is unclear. Perhaps the act of resuspending much of the fine material may permit the survey dredge, a dry dredge, to more effectively bite into the bottom or perhaps the impact of the suction process results in the material not entrained resettling onto the bottom in less consolidated condition. Regardless, the data conform with the previous suggestion of Powell et al. (2002) that some of the variability between dredge efficiency measurements can be explained by the recent history of dredging on the bed.

Interestingly, the dredge efficiency achieved on the control plots rose between July 2002 and May, 2003, as if these plots were impacted by dredging during that intervening period. The Delaware Bay oyster industry carried out its yearly transplant program in August 2002. Reports from participating oystermen indicate that some dredging may have occurred on the control plots at that time. As discussed later, additional information is available from this study that supports the verity of these reports.

Effect of Dredging on the Nursery Bed Population

The transplant efforts resulted in the removal of 11% to 27% of the market-size oysters estimated to have been present on these plots prior to transplant. The suction dredge operated more evenly over the entire plot and at minimal swept area coverage. The dry-dredge boats concentrated on selected sub-areas of the plots and at a substantially higher swept-area coverage. All transplant resulted in the removal of a quantity of animals at or above the level permitted in the present transplant program operated by the industry, a level set at 10%.

Obtaining equivalent population characteristics, even from abutting plots, is unlikely in the patchy world of the oyster bed (Powell et al. 1987, 1995, 2001; Calvo et al. 1996). Our set of plots was not exceptional in this regard. They differed in most population characteristics associated with volumetric or quantitative coverage, although proportional characteristics, such as size class composition, varied infrequently. As a consequence, statistical analyses focused on the degree to which plot attributes changed relative to one another, as revealed by interaction terms between time and treatment.

We examined two time dimensions. The first covered the immediate impact of transplant by comparing the bed just before and just after transplanting. This comparison was partly compromised by the inability to predict *a priori* where the dredge boats would focus their efforts. Consequently, the samples taken prior to dredging did not necessarily reflect the conditions of the dredged portion of the plots. The second time dimension, the succeeding 10 months after transplant, is not encumbered by this uncertainty.

We evaluated the effects of transplant by (1) sampling using a traditional oyster dredge in survey mode to provide volumetric (bushel⁻¹) comparisons, (2) applying dredge efficiency estimates from Table 5 to quantify these data, and (3) using diver samples that were inherently quantitative. Each of these datasets has strengths and weaknesses. The survey tow normally covered a swept area of 90 to 130 m². Such a sample integrates a large area of the bottom, but the sample is not inherently quantitative. Typically, data are expressed on a per-bushel basis as a consequence. Application of an efficiency correction permits quantification, but

also inserts an added degree of uncertainty. In this study, the influence of dredging during transplant seems to have varied dredge efficiency temporally, so that the correction factor varied over time. Diver samples are inherently quantitative, but cover a substantially smaller area; in this case, 3 m² per transect. The five diver transects, collectively, then, account for no more than about 17% of a single dredge-boat sample. Inasmuch as the limitations of each of these sampling methods tend to offset the others, we focused on cases in which the various methods reached corroborative conclusions.

Diver sampling revealed a significant reduction in oyster abundance as a consequence of the transplant, but this reduction could not be confirmed from the dredge samples. The reduction was greatest on the dry-dredge plots, because sampling was concentrated in those areas most heavily dredged and dredging impact was most localized on these plots. Size frequencies were not significantly altered, nor did condition index change more in one treatment type than another. The data suggest only a limited impact of the transplant activity on population attributes.

Sampling over the next 10 months revealed that average size increased and average spat number declined. Condition changed as would be expected by the seasonal cycle in condition (Hopkins et al. 1954, Haven 1962, Versar Inc. 2002). None of these changes occurred to a greater extent in any treatment. Evidence from diver sampling again suggests a differential change in oyster abundance between treatments over time. This trend is corroborated, though less clearly, by significant changes in clump cross-sectional area and the number of oysters per clump in the dredge-boat samples. The overall decline in oyster abundance is partly due to mortality, but also because spat settlement was low in the several preceding years. The significance of the time \times treatment interaction terms accrues from the tendency for oyster abundance and clump size to decline more in the controls than on the dredged plots; however, this, plus the rise in dredge efficiency previously noted, suggests that the August 2002, industry transplant may have impacted the control plots.

Overall, the transplant process had a limited influence on oyster population characteristics on any of the dredged plots, regardless of dredge type. The absence of impact is in keeping with other studies on the physiological effect of dredging on various shellfish, including oysters (Powell et al. 2001, Maguire et al. 2002, Kraeuter et al. 2003), though exceptions exist for more delicate species such as razor clams (Robinson & Richardson 1998).

Bed Complexity

The dry dredge as used for transplant must move a considerable amount of material around the bottom while retaining only a small proportion in the dredge. Presumably, bed patchiness would decline. The suction dredge, with its higher efficiency, should do the opposite. Bed patchiness should increase at a small scale. We examined two spatial scales, the within-diver-transect scale where samples were positioned 2 m apart and the between-diver-transect scale, using a number of measures of between-sample variance. Bed complexity did not change significantly by any measure during the transplant, nor did it change significantly over the subsequent 10 months.

Finally, we looked at the proportion of material contributed by cultch, the single and fragmented valves without live oysters or boxes attached. The concentration factors suggest that the suction dredge removes twice as much or more of this material than the

dry dredge. The proportional contribution of live oysters, boxes, and debris varied between treatments over time. This trend occurred because the volume of live oysters declined noticeably on control plots relative to either treatment over time, in keeping with other indicators that these plots were worked by the industry in August 2002. Cultch volume was not similarly affected, however. The expectation that cultch volume should be affected in some way led to a further examination of the variance between samples on a diver transect. The time \times treatment interaction term was not significant. No evidence exists that transplanting at the level tested in this study results in transient or permanent changes in bed complexity, whether the suction dredge or the dry dredge is used.

Fate of the Transplants

Between 1,000 and 2,500 bushels of material were placed on the New Beds experimental plots. This volume of material did not significantly elevate the abundance of oysters, as measured volumetrically. Evidence of the transplants does manifest itself, however, in a decline in clump cross-sectional area, an increase in the number of oysters per clump, and an increase in condition. Transplants from Cohansey had smaller maximum clump cross-sectional areas, typically 40–60 cm² rather than the pretransplant value of 71 cm², and more oysters per clump, 2–4 clump⁻¹ rather than 0.7 clump⁻¹ on New Beds prior to transplant. This is expected because New Beds oysters consistently average larger than Cohansey oysters (HSRL 2003) and these larger oysters increase clump size. Condition rose from 0.049 g dry wt (cm² shell area)⁻¹ to 0.052–0.068 g dry wt (cm² shell area)⁻¹, but this increase may be due to the elapsed time between sampling before and after transplant, rather than an influence of varying condition between Cohansey bed and New Beds.

Over the next 10 months, the number of oysters declined significantly as Dermo disease took its toll. Year 2002 had the highest natural mortality rate on New Beds and neighboring beds of any year since the incursion of *Perkinsus marinus* into Delaware Bay in 1989 (HSRL 2003). This mortality event affected both suction-dredge and dry-dredge transplants more or less equivalently. Neither condition index nor population size frequency diverged between the two transplant plots, though each varied over time as expected. Thus, to the degree that the sampling program could identify and track the transplants, the two methods of transplanting did not seem to produce divergent results in the fate of the transplanted oysters.

Economics of Transplantation

In this study, the dry-dredge boats loaded 1,053 bushels of material containing an estimated 67,866 market-size oysters at an estimated cost of \$1,579.50 at \$1.50 per bushel. The value per bushel is representative of the cost of industry transplants carried out in Delaware Bay, wherein boats involved in the transplant have received up to \$2 per bushel, depending on the year ([‡]NJDEP, pers. Comm.). The cost of suction dredging is less certain, the record being limited to a single recorded transplant event. In this case, the cost per bushel moved was \$0.70 (NJDEP, pers. comm.). Using this figure, the suction dredge loaded 2,635 bushels in this study containing an estimated 130,828 market-size oysters at an approximated cost of \$1,844.50. A bushel of oysters going to

market contains approximately 345 oysters (HSRL 2003). The dry dredge, therefore, transplanted 196.7 market-equivalent bushels downbay at a cost of \$8.03 per bushel and the suction dredge, 379.2 market-equivalent bushels at a cost of \$4.86 per bushel.

Both cost estimates are likely to be high, in comparison to a normal transplant program, because our Cohansey sites were not chosen to optimize the economics of transplant by targeting areas with the largest densities of market-size animals, and because we have not included in these estimates the survival and growth to market size of smaller oysters. Nevertheless, the comparison is illustrative. The increased efficiency of capture of larger particles by the dry dredge, even taking into account the slower speed at which this was accomplished, offsets in part the increased speed but lower catchability of the suction dredge. Nevertheless, the suction dredge remains about twice as efficient in purely economic terms as the dry dredge.

CONCLUSION

We tested the potential impacts of using a dry dredge and a suction dredge for transplanting at a level anticipated to be at or just above a sustainable level of removal. Over the short time span of a single transplant year, neither method proved deleterious either in its effects on bottom complexity, cultch availability, or population character.

Both dredging styles influenced subsequent dredge efficiency by a traditional dry dredge, probably by reducing bed cohesion, and this change was relatively long-term in duration in that dredge efficiency remained higher 10 months after transplant. However, the activity of dredging does not necessarily impact the oyster populations themselves. Powell et al. (2001) examined the influence of repeated dredging, but were unable to discern any increase in mortality or morbidity in the dredged population. None were detected in this study either. Thus, the increase in dredge efficiency is important in the context of quantitative sampling methodology, and possibly for the economics of fishing, but inconsequential biologically.

Advantageous use of the oyster resource on the upbay nursery beds has 3 requisites. (1) Large animals must be efficiently culled and moved downbay. (2) The upbay nursery beds must be managed to achieve sustainable yield, in this case, sustainable transplant. (3) The process must take place with limited long-term damage to the bed. Given the absence of impact on the portion of the population left on the bed and on bed complexity, these requisites distill to a single principle: market-size oysters should be concentrated during the transplant process, thereby minimizing the number of small oysters and amount of cultch moved downbay. Neither dredging method performed. The suction dredge as configured selectively removes smaller particles enriched in juveniles and cultch. The suction dredge makes inefficient use of the nursery beds and risks the potential for a long-term decline in live oyster abundance and shell coverage. The dry dredge, on the other hand, is inherently capable of concentrating larger animals and the on-deck culling device selectively retains the larger particles. Unfortunately, as used, much of the selective advantage of the dry dredge in transplant disappears. Conceivably, a behavioral shift in the dry-dredging process, by minimizing the time the dredge is left on the bottom, could retain the inherently desirable selectivity traits of the dry dredge in the transplanting process. This same

[‡] New Jersey Department of Environmental Protection

change could minimize the economic disparity presently enjoyed by the suction dredge.

ACKNOWLEDGMENTS

Special thanks to Sam Elias, Captain of the *F/V Howard W. Sockwell* and *F/V Jeanne Christine*, Lou Shultz, Captain of the *F/V Mabel Hollinger*, and Scott Sheppard, Captain of the *F/V Robert Bould*, for their participation in this study. Special thanks also to Royce Reed, Russ Babb, and Jason Hearon of the New Jersey

Department of Environmental Protection for providing vessel and diving support. We particularly thank the dive team headed by Joe Dobarro who collected the 1,080 diver samples that constituted the quantitative samples for this study. Thanks also to Heather Diviney, Bruce Muller, Jeff Gendek, and Allison Pesselano for help in sample analysis. This study was supported by an appropriation from the State of New Jersey through Rutgers University under the oyster stock assessment program managed by the Delaware Bay Section of the Shell Fisheries Council.

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ORGANISMS ASSOCIATED WITH OYSTERS CULTURED IN FLOATING SYSTEMS IN VIRGINIA, USA

F. X. O'BEIRN,* P. G. ROSS AND M. W. LUCKENBACH

College of William and Mary, Virginia Institute of Marine Science, Eastern Shore Laboratory,
P.O. Box 350, Wachapreague, Virginia 23480

ABSTRACT The number and abundance of macro-faunal taxa was estimated from six floating structures (floats) used to culture the Eastern oyster (*Crassostrea virginica*) near Chincoteague Island, Virginia, USA. After a 10-mo grow-out period, all organisms found among and attached to the cultured oysters were counted. The final mean size of oysters was 80.5 (14.7 SD) mm. Overall, 45 species of macrofauna were recorded with the number of species in the floats ranging from 24 to 36. There was no relationship between the number of taxa and the density of oysters in the floats. Total abundances of associated organisms were estimated at 12,746/float to 92,602/float. These findings highlight the diverse (taxonomic and trophic) and abundant nature of communities associated with cultured oysters. They also provide a baseline set of information that may help more clearly define the interactions between oyster culture and the environment.

KEY WORDS: *Crassostrea virginica*, oysters, aquaculture, epifauna, floats

INTRODUCTION

A consequence of declining wild Eastern oyster (*Crassostrea virginica*) stocks throughout the eastern seaboard of the United States (MacKenzie et al. 1997) has been concerted efforts focused on oyster restoration (see Luckenbach et al. 1999a). Shellfish restoration throughout the United States is driven by a number of motives (e.g., ecological and fishery value) and typically involves a wide range of user groups (da Silva Pinho 2000, Breitburg et al. 2000, Brumbaugh et al. 2000, Mann 2000). One approach in support of shellfish restoration is the promotion of aquaculture (mostly of oysters) with the ultimate goal of reducing fishing pressure on wild stocks. During the culture process, oysters are subject to colonization by a variety of organisms. However, the number and relative abundances of taxa associated with the cultured oysters has rarely been assessed, as is the influence of the culture activity on the surrounding habitat. The primary goals of this study are, to identify and enumerate the number of taxa (macro-fauna >2 mm in size) associated with oysters (under culture conditions in Virginia) and to quantify the relative proportions of each taxa. Furthermore, it is hoped that these data will help define any ecological and environmental associations of oysters under culture conditions.

MATERIALS AND METHODS

Oyster culture in Virginia has three distinct stages. The first involves broodstock conditioning, spawning, larval culture, settlement, and an early nursery phase that is effected in a land-based hatchery. Upon removal from the hatchery, a field nursery phase follows where the oysters (3-mm shell height) are placed in mesh bags (2500 oysters per 61 cm × 61 cm bag, 1.5 mm mesh size). As the oysters increase in size, they are stocked in bags with increasingly larger mesh sizes (e.g., 1.5 mm to 3 mm to 9 mm bags) with a concomitant decrease in densities (2500 to 1200 to 600 or less oysters per bag, respectively). The majority of this nursery phase is conducted in off-bottom floating structures called oyster floats (Luckenbach et al. 1999b). The third phase or grow-out phase is conducted either in tray structures located on or near the bottom of

the leased area or in floats. The floats most commonly used are mesh baskets (mesh size = 2.54 cm) that are 2.5 m L × 0.6 m W × 0.3 m D. A polyvinyl chloride (PVC, 10 cm in diameter) collar is secured around the open part of the basket providing floatation for the system. A shade cloth cover is stretched over the top of the float to reduce algal fouling and predation of the single oysters in the floats from birds and mammals (e.g., seagulls and otters). Typically, the oysters are emptied directly into the float for final grow-out (1200–1400 oysters per float). This reduces maintenance associated with tending bags and reduces potential growth restrictions on the oysters, a consequence of confinement within bags. Grow-out typically extends from 8–14 mo. During this period, although the aquaculturist may remove the floats from the water to remove dead oysters, the oysters remain undisturbed until they are harvested.

Oysters for this study were cultured at a polyhaline site south (1 km) of Chincoteague Island, Virginia. In May 1998, oysters were removed from the hatchery at a mean shell height of 3 mm and as part of a field nursery phase were grown as outlined earlier. The grow-out phase commenced early September 1998. As part of another study, examining the effect of differential stocking densities on growth in the floats, three floats were initially stocked with 1750 oysters per float and three with 1000 oysters per float. Dead oysters were removed from the floats twice during the grow-out period (November 1998 and April 1999). After approximately 10 mo (mid June 1999), when it was expected that many of the oysters would be harvestable (at 76-mm shell height), sampling of the oysters and associated fauna took place.

Prior to removal of the floats from the water, a mesh basket (capture basket: 2-mm mesh size) was carefully placed in the water surrounding each float and the capture basket and float were loaded onto the support vessel. The purpose of the exterior basket was to retain any motile fauna that passed through the larger mesh of the float. All motile organisms captured on the exterior basket and located within the float were identified and enumerated. Sampling was effected by blindly selecting 50 oysters from all oysters (within each float) laid out in trays on the deck of the vessel. The shell height and width was measured for each randomly collected oyster. In addition, all attached faunal organisms were identified and enumerated. Colonial species were enumerated as a single

*Corresponding Author. E-mail: francis.obeir@marine.vi

representative of that organism. Total counts of live oysters in the floats were also calculated, thus allowing us to standardize the abundance of organisms in the float.

RESULTS

The final density of oysters cultured in the floats varied considerably and ranged from 488–1381 oysters per float, with an overall mean of 974 oysters per float (Table 1). The density disparity was most likely due to a combination of error associated with original stocking density and differential mortality of oysters in the floats. However, there was little relationship ($r^2 = 0.182$, $P > 0.05$) between oyster density and the number of taxa found in the floats (Fig. 1A). There was a slightly stronger relationship ($r^2 = 0.238$, $P > 0.05$) between the oyster density and the abundance of organisms in the float (see Fig. 1B). Shell height of oysters in the floats ranged from 76.17 to 83.52 mm, with an overall mean of 80.50 mm (see Table 1). The number of macrofaunal taxa in each float ranged from 24 to 36 with an overall mean of 29. In total, 45 faunal taxa were found in the oyster floats (Tables 1 and 2). These represented 11 broad taxonomic groups (see Table 2). The most abundant taxonomic group was annelids that comprised from 47.44% to 69.37% of organisms in the six floats. Mollusks comprised the next most abundant group (11.45% to 27.93%) followed by crustaceans (11.49% to 15.73%). Overall, in terms of abundance, annelids comprised 57.64% of all organisms, mollusks 20.01% and crustaceans 13.76% (see Table 2). These groups comprised 91.4% of all organisms sampled from the floats.

DISCUSSION

The total number of taxa (45) found among the oysters in this study was relatively high and compare favorably with the findings of previous studies of oyster communities (Wells 1961, Bahr 1974, Dame 1979, Larsen 1985, Stanley & Sellers 1986, Zimmerman et al. 1989). Meyer and Townsend (2000) recorded 41 species associated with newly constructed oyster reefs in North Carolina. However, many of these authors report on open reef structures relying on natural recruitment of oysters in mesohaline environments. Given the polyhaline character of the Chincoteague site used in this study it might be expected that the number of taxa recorded would be higher. That greater numbers of taxa were not recorded is likely a consequence of the fact that the assessment of associated organisms was carried out after only 10 months of potential colonization time in an enclosed structure suspended in the water col-

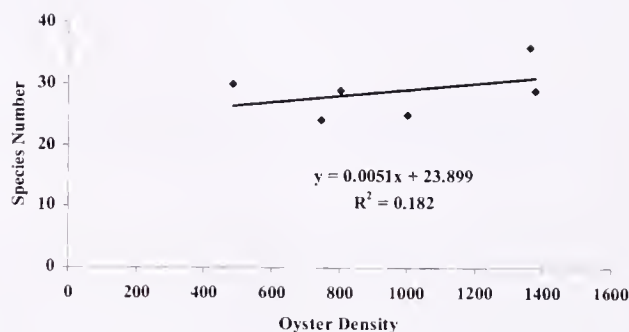
TABLE 1.

Floal individual and overall parameter values calculated for oysters and associated organisms in the culture floats.

		Height	Width		
	Oyster	Mean-mm	Mean-mm	Number	Estimated
	Density	(SD)	(SD)	of Taxa	Abundance
	806	80.98 (14.15)	52.23 (6.29)	29	20,501
	488	80.43 (11.62)	52.33 (8.03)	30	12,746
	749	79.48 (12.20)	51.46 (8.03)	24	28,132
	1363	76.17 (19.56)	47.29 (10.18)	36	92,602
	1004	82.42 (14.54)	51.97 (5.25)	25	32,449
	1381	83.52 (16.55)	51.66 (7.63)	29	36,044
Overall					
means	974	80.50 (14.65)	51.15 (7.62)	~29	37,079

A

Oyster Density vs. Species #



B

Oyster Density vs Abundance

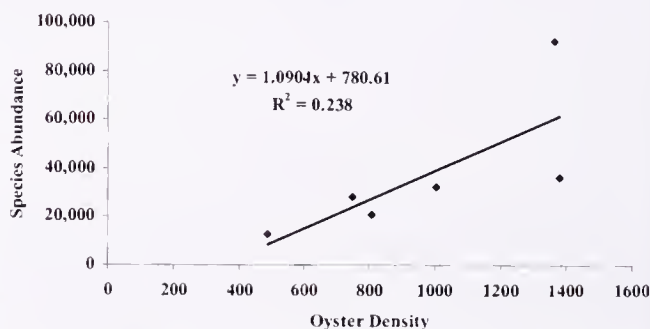


Figure 1. Overall macrofaunal species number plotted against oyster density (A) and overall species abundances plotted against oyster density, in the culture systems (B).

umn. In addition, this limited colonization time may have some bearing on the fact that certain species, typically associated with oysters in similar environments (e.g., the boring sponge, *Cliona celata*) were not recorded in this study.

Palmer et al. (1998) observed that little baseline data are available to serve as guidelines for restoring aquatic habitats. Not the least of these is species composition, from which community structure and trophic interactions may be evaluated. From our findings, a number of trophic groups are apparent in the culture systems. While filter feeders dominated (bivalves), others represented were detritivores (e.g., *Polydora websteri*, *Palaemonetes vulgaris*, omnivores (e.g., *Gobiosoma boscii*) and carnivores (e.g., *Callinectes sapidus*). The diversity of feeding types suggests the potential for various trophic interactions within the culture system and between the system and the surrounding habitat. Of course, limitations are imposed on the interactions by the meshing that comprises the floats. For example, larger predators (e.g., mature blue crabs, fishes, etc.) may be excluded. A potential limitation in interpreting the results of this study, imposed by the sampling methodology, is that smaller motile species (e.g., amphipods) may not have been retained by the 2-mm sampling mesh.

The dominance (in terms of abundance) of relatively few taxa is not unusual for shellfish assemblages. In this study, 3 groups of organisms (polychaetes, crustaceans, and mollusks) comprised 91.4% of all of the macrofauna sampled. Tenore and Gonzalez, (1975) observed that dense epifaunal assemblages (dominated by few taxa) were associated with cultured mussels in Spain. For wild oyster populations, O'Beirn (unpublished data) documented that numerically 94.6% of all faunal organisms found on a constructed oyster reef in Virginia were represented by four taxa. Seed and Suchanek (1992) and Lintas and Seed (1994) had similar findings

TABLE 2.

Relative proportions of organisms sampled in oyster culture floats used in this study and overall means of proportions for each taxa.

		Float						
		Overall Mean	#1	#2	#3	#4	#5	#6
Porifera								
<i>Cliona celata</i>	Boring sponge	0.15		0.22	0.18	0.05	0.17	0.62
<i>Halichondria bowerbanki</i>	Bread sponge	0.81	0.08	0.33	1.07	1.06	0.59	1.09
<i>Lissodendoryx</i> sp.	Garlic sponge	0.06		0.33				0.31
<i>Microciona prolifera</i>	Red beard sponge	0.07			0.18		0.08	0.31
Cnidaria								
Actinaria spp.	Anemones	2.50	0.31	0.89	0.09	4.55	0.08	4.52
Hydroida		3.16	2.59	4.12	3.28	2.90	3.29	3.90
Platyhelminthes								
<i>Stylochus ellipticus</i>	Flatworm	<0.01		0.01				
Annelida								
<i>Hydroides dianthus</i>	Fan-worm	25.99	24.69	20.28	28.84	24.19	33.96	22.93
<i>Lepidonotus</i> sp.	Scale worm	<0.01	0.01	0.01		0.01		
<i>Polydora websteri</i>	Spionid-worm	<0.01	0.01	0.01			0.01	0.01
<i>Sabella microphthalmia</i>	Fan worm	1.32	0.39	0.22	0.71	2.71	0.25	0.31
<i>Sabellaria</i> sp.	Mason worm	0.26			0.18			2.03
<i>Spirorbis</i> sp.	Spiroid worms	29.88	34.44	32.53	39.76	26.58	31.93	22.15
Crustacea								
<i>Balanus eburneus</i>	Barnacle	11.46	12.74	10.58	8.08	12.83	12.50	7.64
<i>Callinectes sapidus</i>	Blue crab	0.01	0.01	0.01	0.01	0.01	0.01	0.01
<i>Cronius ruber</i>	Red crab	<0.01	0.01	0.01		0.01		
<i>Dyspanopeus sayi</i>	Mud crab	0.16	0.20	0.21	0.30	0.10	0.16	0.16
<i>Eurypanopeus depressus</i>	Mud crab	0.02	<0.01	0.01	0.01	0.03	0.01	0.01
<i>Hemigrapsus sanguineus</i>	Asian shore crab	<0.01	<0.01					
<i>Lyssmata wurdemannii</i>	Peppermint shrimp	<0.01				<0.01		
<i>Palaemonetes vulgaris</i>	Grass shrimp	1.73	0.27	4.33	3.61	1.43	0.52	2.99
<i>Xanthidae</i> spp.	Xanthid crabs	0.12		0.23	0.20	0.04	0.28	0.18
<i>Panopeus herbstii</i>	Mud crab	0.26	0.21	0.35	0.33	0.17	0.28	0.50
Mollusca								
<i>Anadara ovalis</i>	Blood ark	0.01		0.11				
<i>Anadara transversa</i>	Transverse ark	0.02				0.05		
<i>Anomia simplex</i>	Common jingle	3.77	2.75	4.90	1.60	3.77	2.53	8.11
<i>Crassostrea virginica</i>	Eastern oyster	15.47	18.95	16.49	9.76	16.83	11.49	17.16
<i>Crepidula fornicata</i>	Slipper shell	0.20	0.31	0.11	0.09	0.28	0.08	0.16
<i>Crepidula plana</i>	Slipper shell	0.49	0.08	0.56		0.41		2.34
<i>Doris verrucosa</i>	Nudibranch	<0.01		0.01		<0.01		
<i>Mytilus edulis</i>	Blue mussel	0.04		0.11		0.05		0.16
<i>Urosalpinx cinerea</i>	Oyster drill	<0.01					<0.01	
Bryozoa								
<i>Membranipora tenuis</i>	Encrusting bryozoan	1.30	1.10	1.45	1.24	1.38	0.93	1.72
Echinodermata								
<i>Arbacia punctulata</i>	Purple sea urchin	<0.01				<0.01		
Tunicata								
<i>Aplidium stellatum</i>	Sea pork	0.06	0.08	0.56				0.16
<i>Didemnum</i> sp.	Paintsplash tunicate	0.05	0.08			0.05		0.16
<i>Mogula manhattensis</i>	Sea squirt	0.09	0.08	0.67	0.18	0.05		
<i>Styela plicata</i>	Rough sea squirt	0.16	0.16	0.33		0.14	0.17	0.31
Pisces								
<i>Chasmodes bosquianus</i>	Striped blenny	<0.01	0.02			<0.01		
<i>Fundulus heteroclitus</i>	Mummichog	0.13	0.09		0.16	0.07	0.43	0.05
<i>Gobiosoma boscii</i>	Naked goby	0.02	0.03			0.04	0.01	
<i>Gobiosoma strumosus</i>	Skilletfish	0.01	0.02		0.01	0.01	<0.01	
<i>Opsanus tau</i>	Oyster toad fish	<0.01				<0.01		
<i>Hypsoblennius hentzi</i>	Feather blenny	0.18	0.28	0.01	0.14	0.23	0.22	0.01
<i>Tautoga onitis</i>	Tautog	<0.01				<0.01		

for faunal communities associated with the blue mussel, *Mytilus edulis*. It must be noted that, whereas numerical dominance (as assessed in this study) may be a useful indicator of community composition, species biomass (not assessed in this study) may

yield different conclusions regarding community structure that may be equally as important.

The occurrence of some species in the floats is of particular interest. The portunid crab, *Cronius ruber*, was considered rare in

Virginia (Van Engel & Sandifer 1972). However, in this study, five specimens, ranging in size from 24.9–34.9 mm, were located within the floats. These findings allied with previous and subsequent observations (O'Beirn personal observation), suggest that they are more than just occasional or rarely occurring species. The numerous fish species sampled highlight the importance of oysters as habitat for these species. Not only is the interstitial space among the oysters important, but dead articulated shells also provide useful habitat (in the form of refuge or nesting sites) for the blennies and gobies (Breithurg 1999).

The increase in aquaculture activities throughout the world has initiated much discussion and research on the interaction between the activity and the environment (Freeman 1988, Hickey & Hurst 1989, Iwama 1991, Hastings & Heinle 1995). Oysters may be cultured in areas that were previously devoid of the species. Hence, they could be regarded as an introduced species into a system. Simenstad and Fresh (1995) recommend that resource managers should consider the effects of aquaculture and attendant activities on ecosystem and community levels. The effects of introducing a large-scale monospecific culture operation into a particular system can have a cascading effect and ultimately affect production and the species composition inherent within the system (Simenstad & Fresh 1995). A shift in the ecological balance may affect the ability of the communities within the system to withstand perturbations of one form or another.

It has been suggested that shellfish culture activities benefit the environment by attracting numerous other organisms to the area (Dewey 2000). Whether they are truly an ecological or environmental benefit or not has yet to be specifically determined. The benefits (i.e., habitat and production) afforded by oyster culture activities may be minimal given the ephemeral nature of these communities (mediated by harvest and handling schedules). Many organisms constituting these communities may not actually mature

to reproduce. Consequently, these systems may be regarded as sink populations. In addition, certain associated species may actually compete with the culture organism for food resources (e.g., blue mussels and wild oysters setting on the cultured oysters; Adams et al. 1991), and increase the organic loading emanating from the culture systems (Nugues et al. 1996). Finally, as urbanization and human activities lead to an increase in habitat alteration in coastal areas (Connell 2000), suites of potentially competing organisms may be introduced into an area. Such a phenomenon may upset the ecologic balance of the area by changing the constituent organisms and species interactions within the system. As the scale of anthropogenic activities increase, it will be increasingly important to further elucidate these and other ecologic interactions.

A major impediment to successful restoration of impacted shellfish habitats is defining success of a particular venture (Coen & Luckenbach 2000). Defining goals and establishing success criteria has proven difficult, given that in many regions natural shellfish habitats (to use as reference locations) are lacking (Luckenbach et al. 1999a, and references therein). Anecdotal accounts of shellfish population structure and associated organisms in particular regions most often are insufficient upon which to base an expensive and long-term restoration program. Therefore, these data may give an indication (in terms of community assemblage and/or trophic interactions) of what may be expected when initiating an oyster restoration program.

ACKNOWLEDGMENTS

The authors thank the staff and students of the VIMS Eastern Shore Laboratory who helped in this study. Helpful reviews of two anonymous reviewers are also duly acknowledged. This paper is Contribution No. 2619 of the Virginia Institute of Marine Science, The College of William and Mary.

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PARASITIC AND SYMBIOTIC FAUNA IN OYSTERS (*CRASSOSTREA VIRGINICA*) COLLECTED FROM THE CALOOSAHATCHEE RIVER AND ESTUARY IN FLORIDA

JAMES T. WINSTEAD,¹ ASWANI K. VOLETY² AND S. GREGORY TOLLEY²

¹United States Environmental Protection Agency, 1 Sabine Island Dr., Gulf Breeze, FL 32561, ²Florida Gulf Coast University, 10501 FGCU Blvd. South, Fort Myers, FL 33965

ABSTRACT Studies of oysters, *Crassostrea virginica*, collected from 10 sites in the Caloosahatchee River and Estuary, Florida, revealed a varied parasite and symbiotic faunas that have never been reported in this area. Organisms observed included ovacystis virus infecting gametes at four sites (prevalence <1%), ciliate protozoans *Ancistrocoma* sp. in the gut of a stressed oyster at one site and *Sphenophrya* sp. infecting the gills of animals at three sites (prevalence <1%). The gregarine protozoan *Nematopsis* was found at all 10 sites (prevalence 24% to 90%) and oysters at some sites had concurrent infections of *Nematopsis pyrrherchi* and *Nematopsis ostrearium* in connective tissue near the stomach, mantle, and gills. Light to moderate infestations of hydrozoan polyps of a species in the genus *Eutima* were observed in the gills of oysters at all sites (prevalence 1% to 22%). Helminths included an unidentified turbellarian (prevalence 1% to 4%) observed at three sites and the digenetic trematodes *Echinostoma* sp., *Proctoeces maculatus* and *Bucephalus* sp. in oysters at five (prevalence 1% to 93%), three (prevalence >1%), and six (prevalence 1% to 3%) sites respectively. The first two trematodes were found infesting the gonoducts of their hosts while sporocysts of *Bucephalus* sp. infected connective tissues and gonads. Metacercariae of a species in the genus *Tylocephalum* were found in vesicular connective tissues near the gut, mantle, and in the gills of animals at all sites (prevalence 7% to 58%). Many sites had oysters with multiple infestations/infections of the above organisms indicating a rich biotic diversity, especially at those sites least impacted by human activity.

KEY WORDS: oysters, parasite, symbiont, prevalence, diversity

INTRODUCTION

Eastern oysters (*Crassostrea virginica*) are highly valued as food, but their ecological significance is under-appreciated and under-studied (Coen et al. 1999a). The oyster's ability to form large biogenic reefs (Coen et al. 1999b) qualifies it as a keystone or valued ecosystem component (VEC) species. VEC species are those that sustain the ecological structure and function of dominant estuarine communities. These species provide not only food, but also the physical habitat used by other organisms for living space, refuge, and foraging sites (Volety et al. 2003a). The complex 3-dimensional reef structure it forms attracts numerous species of invertebrates and fishes. To date, over 300 species have been identified as depending, either directly or indirectly, on oyster reefs (Wells 1961, Crabtree & Dean 1982, Abbe & Breitburg 1992, Wenner et al. 1996, Coen et al. 1999a) and many of these organisms in turn serve as forage for important fishery species (Tabb & Manning 1961, Marshall 1958, Gilmore et al. 1983) and birds (Watts 1988). Using a suite of responses from oysters, studies of the effects of heavy metals, organochlorine pesticides, rainfall, and freshwater releases into the Caloosahatchee Estuary from Lake Okeechobee on these animals were carried out. In addition, the effects of rainfall and freshwater releases on species abundance, richness, biomass, and diversity in Caloosahatchee oyster reefs were investigated. Data obtained from these studies will be used to assess the anthropogenic impact on the estuary and to provide target conditions for watershed management (Volety et al. 2003a, 2003b). One aspect of these studies, histologic examination of oysters, revealed a number of parasites and symbionts living in and on collected animals. Parasites have been used as an indicator species in environmental assessments for some time. There have

been a number of investigations on the effects of pollution and generalized human disturbance on parasites of fishes and mollusks to indicate anthropogenic impacts (Møller 1987, Khan & Thulin 1991, MacKenzie et al. 1995, Kuris & Lafferty 1994, Lafferty 1997). The most common approach is to compare the prevalence or intensity of parasitism among hosts sampled from a number of control and impact sites or a single site before and after the impact (Lafferty 1997). The rationale being that the diversity and presence of parasites, especially larval parasites and their intermediate hosts, can be potentially useful indicators of disturbances that reduce the abundance and diversity of vertebrates that act as the parasites' final host (Lafferty 1997). Thus, information regarding the presence or absence of parasites/symbionts in the Caloosahatchee estuary should be useful in assessing anthropogenic impacts when combined with all other aspects of the study. Therefore, the objective of this study is to report, for the first time, a survey of those parasites and symbionts observed in oysters from selected collection sites in the Caloosahatchee study area and briefly discuss how their presence or absence may indicate greater biologic diversity and a measure of ecosystem health.

MATERIALS AND METHODS

Sampling Locations

Oysters were examined from two projects being conducted within the same area of the Caloosahatchee estuary (Fig. 1) during a similar time period. The first project was designed to examine the effects of season and freshwater flow on the ecologic and physiologic processes of oysters as well as the habitat use of oyster reefs by fish and crustaceans. Monthly samples, 10 oysters per site (total 239 per site), were taken from 5 collection sites between September 2000 and December 2002. Sample sites included Piney Point (PP, 4 km upstream from the river mouth), Cattle Dock (CD, 2 km upstream from the river mouth), Bird Island (BI, 4 km downstream from the river mouth), Kitchel Key (KK, 6 km downstream from the river mouth), and Tarpon Bay (TB, 12 km downstream from the river mouth). The second project was designed to examine the

Funding for this study was made possible by grants from the South Florida Water Management District and the Charlotte Harbor National Estuary Program, Contribution No. 1206, of the Gulf Breeze Environmental Research Laboratory.

*Corresponding author. E-mail: wintead.jim@epa.gov

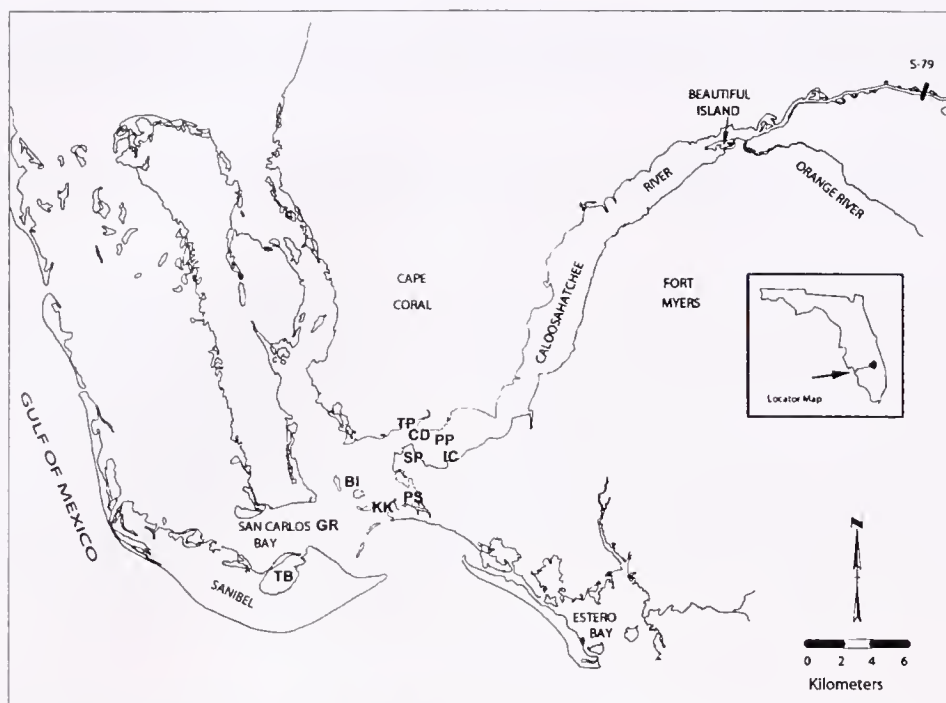


Figure 1. Map of Caloosahatchee River Estuary showing oyster sampling sites. PP, Piney Point; CD, Cattle Dock; BI, Bird Island; KK, Kitchel Key; TB, Tarpon Bay; IC, Iona Cove; TP, Tarpon Point; SP, Shell Point; GR, Greg's Reef; PS, Port Sanibel.

relationship between heavy metals, PCBs, and organochlorine pesticides measured in water and oyster tissues with the physiologic and ecologic responses of sampled oysters. Samples, 10 per site (total 78 to 90 per site), were taken from 5 sites every other month between June 2001 and December 2002. Sample sites included Iona Cove (IC, 4 km upstream from the river mouth), Tarpon Point (TP, 1.5 km upstream from the river mouth), Shell Point (SP, 1.75 km upstream from the river mouth), Port Sanibel (PS, 5 km downstream from the river mouth), and Greg's Reef (RS, 6 km downstream from the river mouth).

Histologic Analysis

Oysters sampled for histology were shucked and transverse whole-body sections (1–2 cm) were cut from immediately behind the labial palps to obtain a representative area of mantle, gills, gonadal, and digestive organs. These tissues were fixed in Davidson's fixative (Shaw & Battle, 1957) and prepared for paraffin sectioning. Sections (7 μ m) were stained with Harris' hematoxylin and eosin and examined via light microscopy for parasites and gonadal reproductive stages.

Data concerning the protist parasite *Perkinsus marinus* and relationships of agents described in this study with the compounds and environmental factors that were measured in the second study will be presented in more detail in a subsequent manuscript.

Definition of Specific Terms

Infection/infestation: "an infection is the invasion or state resulting from an invasion by a parasite or a pathogen into a host. An infestation is an external rather than an internal invasion. If both internal and external organisms occur on one host, all the associations are collectively referred to as infections. Neither an infection nor an infestation necessarily imply disease" (Overstreet 1978).

RESULTS

A polyoma-like virus was found in gametes (eggs) of oysters from Piney Point, Kitchel Key, Tarpon Bay and Port Sanibel stations (Table 1). The viral infection produced abnormal hypertrophied, basophilic ova ranging up to 250 μ m in diameter with scant cytoplasm (Fig. 2). Pathologic response to the condition was limited to a light to moderate hemocytic infiltration that may have been in response to lysis of affected ova. Prevalence was low (<1%) at all stations where the virus was found (Table 1).

A ciliate, *Ancistrocoma* sp., was found attached to the epithelium and free in the lumen of digestive diverticulum in one oyster from the Cattle Dock Station (Table 1). This oyster appeared to be undergoing physiologic stress from *Perkinsus marinus* infection with a significant hemocytic response to the *Perkinsus* and an epithelial lesion in the stomach (Fig. 3). Another larger basophilic ciliate, *Sphenophrya* sp., was found attached to gills of animals collected from Bird Island, Kitchel Key, and Tarpon Bay. This ciliate formed colonies sufficient enough to cause xenomas in all affected oysters (Fig. 4). Prevalence was low at all stations (<1%) and besides the obvious xenomas, no pathologic damage was observed.

Apicomplexan oocysts, *Nematopsis* sp. (Fig. 5) were found in connective tissue of the gills, mantle and digestive diverticulum at all collection sites sampled (Table 1). In some cases, oocysts found in the gills were larger than those in the mantle and digestive diverticulum of the oyster (19 \times 15 μ m vs. 13.4 \times 10 μ m respectively). Infections were light for the most part and no pathologic damage, other than tissue space taken up by the organism, was observed. Average prevalence was greatest at Tarpon Bay (92%, range 70% to 100%) and Piney Point stations (90%, range 20% to 100%) and lowest at Iona Cove (24%, range 0% to 70%).

Hydrozoan polyps of a species in the genus *Eutima* sp. (Fig. 6)

TABLE 1.

Prevalence of parasites and symbionts in oysters (*Crassostrea virginica*) collected from 10 stations within the Caloosahatchee River/Estuary study area. Values in parentheses represent prevalence (% of parasite/symbiont presence).

Parasite/Symbiont	Piney Point	Cattle Dock	Bird Island	Kitchel Key	Tarpon Bay	Iona Cove	Tarpon Point	Shell Point	Gregg's Reef	Port Sanibel
<i>Ovacystis</i> virus	1/239 (0.4%)	0/239 (0%)	0/239 (0%)	1/238 (0.4%)	1/238 (0.4%)	0/80 (0%)	0/90 (0%)	0/90 (0%)	0/90 (0%)	1/78 (1%)
<i>Ancistrocoma</i> sp.	0/239 (0%)	1/239 (0.4%)	0/239 (0%)	0/238 (0%)	0/238 (0%)	0/80 (0%)	0/90 (0%)	0/90 (0%)	0/90 (0%)	0/78 (0%)
<i>Sphenophrya</i> sp.	0/239 (0%)	0/239 (0%)	1/239 (0.4%)	1/238 (0.4%)	0/238 (0.4%)	0/80 (0%)	0/90 (0%)	0/90 (0%)	0/90 (0%)	0/78 (0%)
<i>Nematopsis</i> spp.	214/239	168/239 (70%)	158/239 (66%)	172/238 (72%)	218/238 (92%)	19/80 (24%)	25/90 (28%)	53/90 (59%)	73/90 (81%)	49/78 (63%)
<i>Eutima</i> sp.	10/239 (4%)	52/239 (22%)	8/239 (3%)	1/238 (0.4%)	3/238 (1%)	8/80 (10%)	8/90 (9%)	14/90 (16%)	3/90 (3%)	12/78 (15%)
Turbellaria	0/239 (0%)	0/239 (0%)	10/239 (4%)	6/238 (2.5%)	3/228 (1%)	0/80 (0%)	0/90 (0%)	0/90 (0%)	0/90 (0%)	0/78 (0%)
<i>Echinostoma</i> sp.	0/239 (0%)	6/239 (3%)	30/239 (13%)	3/238 (1%)	222/238 (93%)	0/80 (0%)	0/80 (0%)	0/80 (0%)	50/90 (56%)	0/80 (0%)
<i>Proctocox</i> sp.	0/239 (0%)	1/239 (0.4%)	0/239 (0%)	0/238 (0%)	0/238 (0%)	0/80 (0%)	0/90 (0%)	1/90 (1%)	0/90 (0%)	1/78 (1%)
<i>Bucephalus</i> sp.	8/239 (3%)	0/239 (0%)	2/239 (1%)	2/238 (1%)	0/238 (0%)	1/80 (1%)	1/90 (1%)	0/90 (0%)	0/90 (0%)	1/78 (1%)
<i>Tylocephalum</i> sp.	74/239 (31%)	72/239 (30%)	115/239 (48%)	98/238 (41%)	138/238 (58%)	6/80 (8%)	23/90 (26%)	6/90 (7%)	48/90 (53%)	26/78 (33%)

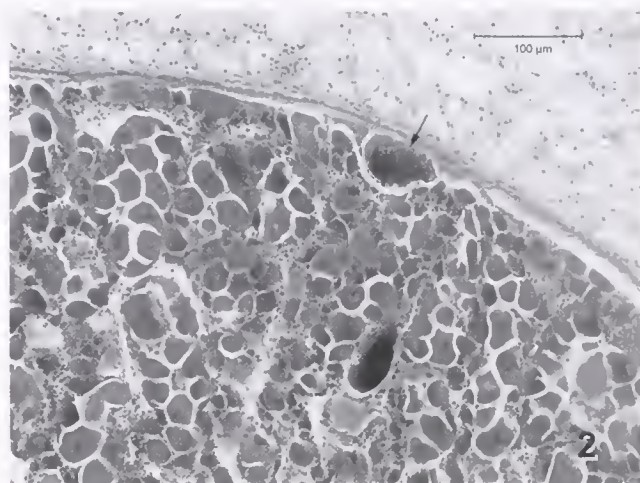


Figure 2. Light micrograph showing normal and virus-infected hypertrophied ova (arrow) in the gonad of an oyster, *Crassostrea virginica*.

were found infesting the gills of oysters at all collecting sites (Table 1). Polyps were attached to gill epithelium and most infestations appeared to be light to heavy (3–5 animals per histologic section). The hydroid's pedal disc did not appear to penetrate the oysters epithelium and no observable pathology was detected beyond possible obstruction of water flow to the gills. Highest prevalence was seen at Cattle Dock (22%, range 0% to 80%) and Shell Point (16%, range 0% to 80%) whereas Kitchel Key and Tarpon Bay had the lowest numbers (<1%).

An unidentified turbellarian (Fig. 7) was found infesting the gills of oysters at three stations (Table 1). Worms were closely associated with gill epithelia and infestations appeared to be light (1–3 worms per histologic section). These organisms had an elongate, ciliated body 0.081 to 0.162 mm (mean, 0.107) wide by 0.112 to 0.25 mm (mean, 0.17) long. The worms elicited a moderate hemocytic response in the oysters' gills and many became engulfed in copious mucous secreted from and surrounding the worm (see Fig. 7). Prevalence was greatest at Bird Island (4%) whereas Kitchel Key and Tarpon Bay were 2.5 and 1%, respectively (Table 1).

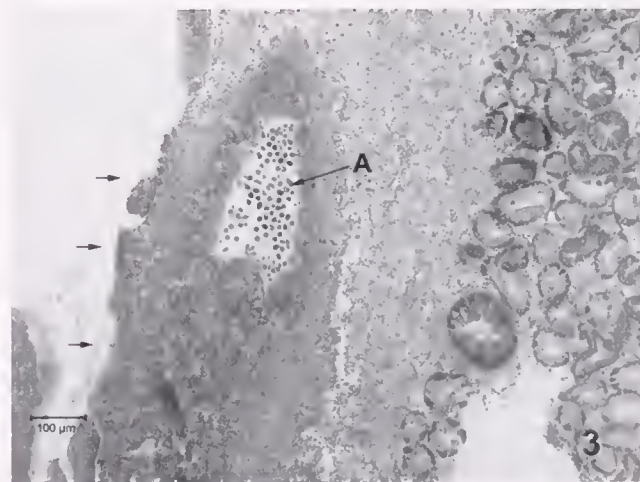


Figure 3. Ciliate infection (A), *Ancistrocoma* sp., in the gut of a stressed oyster, *Crassostrea virginica*. Note damaged epithelial lining of the gut (arrows) with concomitant hemocytic response to damage.

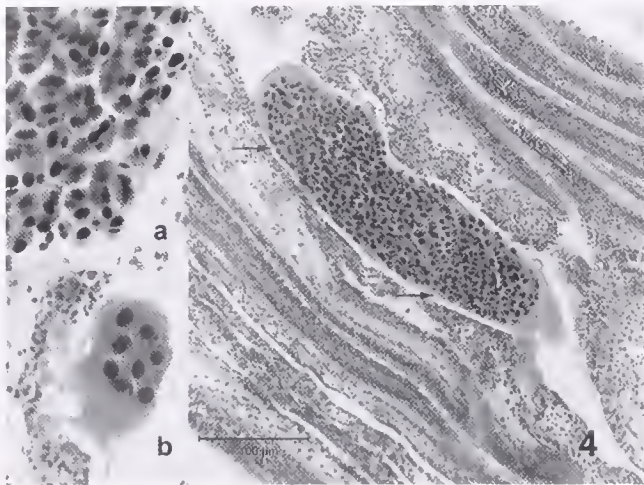


Figure 4. Ciliate infection, *Sphenophrya* sp., in the gills of an oyster, *Crassostrea virginica*, creating a xenoma, seen as numerous dark nuclei (arrows). Note normal gill tissue to the left and right. Insert (a) shows a higher magnification of 4. Insert (b) shows the beginning of xenoma formation in gills of a different oyster.

Large numbers of encysted metacercaria of a digenetic trematode species in the genus *Echinostoma* (Fig. 8) were found encysted in the gonoducts of oysters from five collection sites (Table 1). Morphologic features are similar to trematodes reported in oysters (*C. virginica*) from Tampa Bay, Florida (Fisher et al. 1996, Winstead et al. 1998). The helminths did not appear to cause any tissue damage but localized massive hemocytic responses to necrotic worms were quite evident (see Fig. 8). Average prevalence was greatest at Tarpon Bay (93%, range 20% to 100%) and Greg's Reef sites (56%, range 0% to 100%) and lowest (of those collection sites with the parasite) at Kitchel Key (1%, range 0% to 10%).

Unencysted metacercarial stages of another digenetic trematode species in the genus *Proctoeces* (Fig. 9) were found in the gonadal follicles and gonoducts of male and female oysters from Shell Point, Port Sanibel and Cattle Dock sites (Table 1). General morphologic features of the sectioned trematode are similar to *Proctoeces maculatus* described in oysters, *C. virginica*, from northern Gulf coast estuaries (Winstead & Couch 1981). Oysters



Figure 5. Oocysts of a species in the genus *Nematopsis* infecting the vesicular connective tissue in the gills of the oyster, *Crassostrea virginica*.

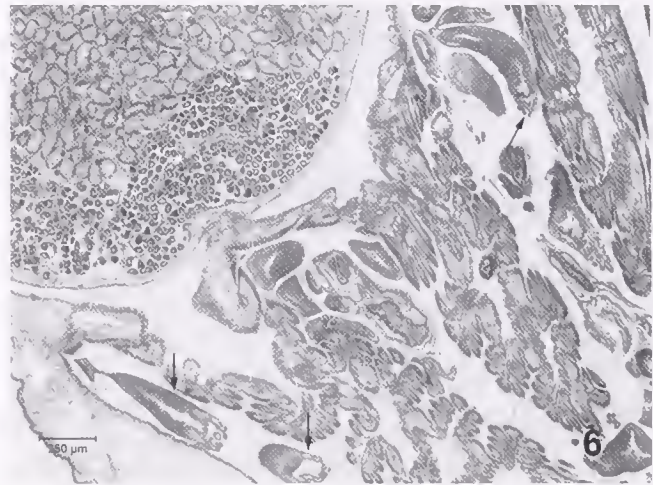


Figure 6. Polyps of a hydrozoan in the genus *Eutima* infesting the gills of the oyster, *Crassostrea virginica* (arrows).

showed no significant hemocytic or other cellular reaction to the worm. Also, even though the observed worms were surrounded by oyster gametes (see Fig. 9), there was no evidence of gonadal impairment to the oyster.

Sporocysts of a species of digenetic trematode in the genus *Bucephalus* (Fig. 10) were found in vesicular connective tissue of gills, palps, digestive diverticula, and in the gonads of male and female oysters from Piney Point, Bird Island, Kitchel Key, Iona Cove, Tarpon Point, and Port Sanibel (Table 1). Sporocysts contained numerous germ balls and developing cercaria (see Fig. 10). Many animals had heavy infections in which branching sporocysts of the parasite had completely destroyed the gonads by mechanical damage and physiologic decomposition of gametes. There was little host response to the presence of the worm except in instances where hemocytic reaction to dead or degenerate sporocysts was evident. Piney Point had the greatest prevalence (3%) whereas the other collection sites, where the parasite was observed, showed less than 1% infection.

Metacystodes of a species of cestode in the genus *Tylocephalum* (Fig. 11) were observed infecting vesicular connective tissue

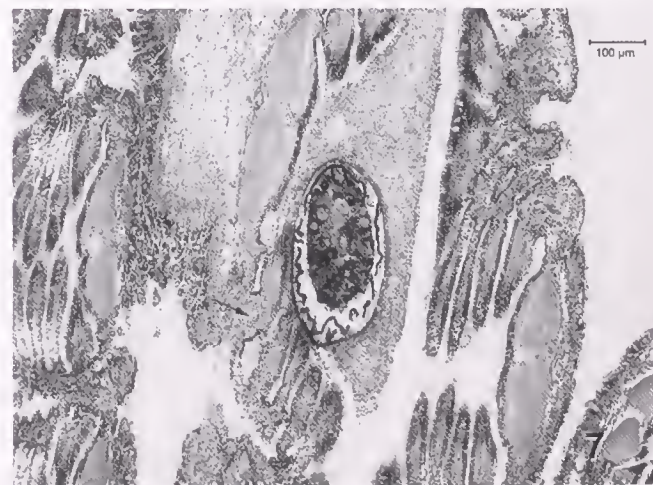


Figure 7. Unidentified turbellarian infesting gills of oyster, *Crassostrea virginica*. Note moderate hemocytic reaction in gills and copious mucus secretion, filled with oyster hemocytes, surrounding the worm.

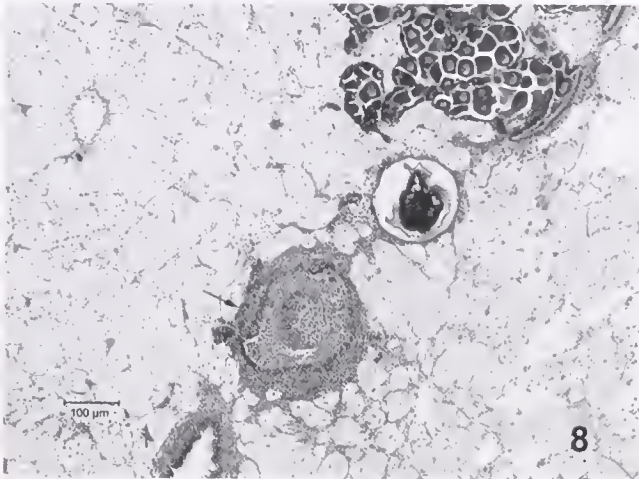


Figure 8. Encysted metacercariae of a digenetic trematode in the genus *Echinostoma*, in the gonoduct of the oyster, *Crassostrea virginica*. Note heavy hemocytic response to dead trematode in adjacent gonoduct (arrow).

in gills, digestive diverticula, and stomach at all collection sites (Table 1). Larval worms elicited a host response, which included a thick encapsulation consisting of a fibrocyte-like inner layer and an outer hemocytic response that appeared more severe around those worms closest to the stomach (see Fig. 11). Some of the parasites appeared to be undergoing resorption by the host. Also, many oysters had multiple infections of the parasite (see Fig. 11). Average prevalence of the worm was greatest at Tarpon Bay (58%, range 30% to 90%) and Greg's Reef (53%, range 20% to 90%) whereas Iona Cove (8%, range 0% to 30%) and Shell Point (7%, range 0% to 20%) had the lowest.

Concurrent infestations in many oysters by two to five of the parasites described earlier in the discussion were observed at all collection sites. This was especially evident at Tarpon Bay and Gregs Reef (Table 1) where many oysters had *Nematopsis* sp., *Tylocephalum* and *Echinostoma* sp. and some at Tarpon Bay were also infested with turbellarians and hydroid polyps.

DISCUSSION

The polyoma-like virus infecting the gametes of some Caloosahatchee oysters caused massive hypertrophy of infected eggs but,



Figure 9. Unencysted metacercariae of a digenetic trematode, *Proctoeces maculatus*, in the gonoduct of the oyster, *Crassostrea virginica*.

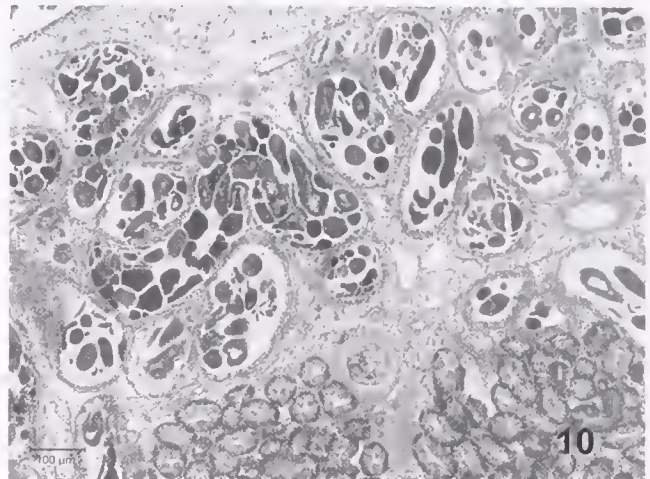


Figure 10. Sporocysts, filled with cercariae and germ balls, of a digenetic trematode in the genus *Buccaphalus* invading and replacing the gonad of the oyster, *Crassostrea virginica*.

other than a light to moderate hemocytic infiltration into localized areas where infected eggs were, did not appear to cause any significant pathologic response. This condition has been described in *C. virginica* from the Piscataqua River, Maine (Farley 1976) Long Island, New York (Meyers 1981) and the northeastern Gulf of Mexico (Winstead et al. 1998, Winstead & Courtney 2003). Similar lesions have been reported in *C. gigas*, *C. commercialis*, *Ostrea lurida*, and *O. edulis* (Farley 1978, Lauckner 1983). The condition has been termed "ovacystis disease" and is characterized by massive hypertrophy of ova and gametocytes, which contain Feulgen-positive (DNA) granular masses in the nucleus (Farley 1976, Winstead & Courtney 2003). Whereas normal ova reach a maximum of 75 μm in diameter (Galtsoff 1964), hypertrophied ova can reach up to 500 μm in diameter. Electron microscopy in the earlier cases revealed the presence of viral-like particles within the nuclei of hypertrophied cells. Prevalence of the condition was low and oysters showed little pathologic effects as was the case in the Caloosahatchee oysters.

The Ancistrocomid ciliates found in the gut of one oyster at



Figure 11. Metacystodes of a cestode in the genus *Tylocephalum* in the vesicular connective tissues of the oyster, *Crassostrea virginica*. Note the heavy fibrous encapsulation around larval cestodes by the oyster.

Cattle Dock Station were probably responding to the injured oyster as secondary invaders rather than as primary pathogens. The lesion in the oyster's gut concomitant with the heavy infection of *P. marinus* probably weakened the oyster and made it more susceptible to these invaders. Ancistrocomid ciliates have been reported in numerous bivalves from the Atlantic, Pacific, and Gulf coasts (Lauckner 1983). In the Gulf of Mexico, what were probably ancistrocomid ciliates have been reported infecting the gut and digestive diverticula in oysters from Pascagoula Bay, Mississippi, Mobile Bay, Alabama, Pensacola Bay, Florida (Couch 1985), and Louisiana estuaries (Mackin 1962, Gauthier et al. 1990). Couch (1985) and Gauthier et al. (1990) reported little pathology associated with these infections. However, Mackin (1962) concluded that these ciliates may become a complicating factor when oysters are infected with *Dermocystidium marinum* (= *Perkinsus marinus*) and Burton (1963) reported ancistrocomid ciliates associated with an abnormal histologic appearance of the host's tissues and unusual concentrations of hemocytes in "gaper" oysters from Chesapeake Bay, Maryland. The condition of the Caloosahatchee oyster, *Perkinsus* infection with massive hemocytic response to the parasite and an injury, appear similar to those in oysters described by Mackin (1962) and Burton (1963). This would lend credence to Lauckner's (1983) conclusions that ancistrocomids, normally gill parasites that cause little pathology to their host, in some cases become opportunistic and invade the host's tissues under abnormal conditions of physiologic stress.

Sphenophrya sp., ciliates induced the formation of xenomas in the gills of affected oysters. Some oysters had multiple infection sites in their gills. Infected epithelial cells contained anywhere from 3 to 4 to hundreds of ciliate nuclei per site. Aside from the possibility of occluding water tubules in the gills, no observable pathology was detected. *Sphenophrya*-like ciliates have been reported in the gills of *C. virginica* from the Atlantic coast (Newman 1971, Otto et al. 1979, Meyers 1981), and in the Gulf of Mexico, similar ciliates have been observed infecting the gills in oysters from Pascagoula Bay, Mississippi, Mobile Bay, Alabama, Pensacola Bay, Florida (Couch 1985), and coastal Louisiana (Gauthier et al. 1990). Sphenophryids are unique because when they occur in large numbers they can induce a distinctive lesion known as a xenoma (Weissenberg 1922). This condition ensues after one of the organisms enters a host cell and proliferates, stimulating the host cell to undergo repeated karyokinesis and massive hypertrophy to accommodate the numerous ciliates contained therein. Xenomas range from the size of a single cell to a macroscopic cell about 3 mm long (Otto et al. 1979) and, as discussed, this condition was observed in all the Caloosahatchee oysters found to be infected with the ciliate. The occurrence of these organisms, whether a single sphenophryid or in xenoma form, has not been reported to cause distress to infected oysters (Lauckner 1983).

The Caloosahatchee oysters exhibited a high prevalence of *Nematopsis* from most collection sites. Most infections were light and, aside from taking up tissue space in the gills or visceral mass, did not appear to cause any pathologic damage to their hosts. Many oysters had two different sizes of oocysts located either in vesicular connective tissue around the stomach and mantle or in the gills. Gregarines of the genus *Nematopsis* use *C. virginica* as a normal intermediate host and are quite common in many different species of bivalves along the Pacific, Atlantic, and Gulf coasts (see review by Lauckner 1983). More recent studies have reported the parasite in oysters from Pascagoula Bay, Mississippi; Mobile Bay, Alabama; Pensacola Bay, Florida (Couch 1985); coastal Louisiana

(Gauthier et al. 1990); Apalachicola Bay, Florida (Fisher et al. 1996); and Tampa Bay, Florida (Fisher et al. 2000). The presence of different size oocysts parasitizing a number of the same oysters at different sites probably indicates a concurrent infection by two different species of this protist. The smaller size oocysts from the Caloosahatchee oysters are probably those of *N. oostreum* because they are both similar in size ($\sim 10 \times 14 \mu\text{m}$) and are most conspicuous in the mantle and in connective tissue around the digestive diverticula, where *N. oostreum* are generally found (Sprague 1949, Lauckner 1983). Also, numerous mud crabs *Panopeus* sp. and *Eurypanopeus depressus*, which are the definitive hosts for this parasite (Lauckner 1983), were abundant at all study sites where the protist was observed (Volety et al. 2003a). The larger oocysts are probably those of *Nematopsis prytherchi* because they are similar in size ($\sim 19 \times 16 \mu\text{m}$), occur in the gills, and have been reported to occur concurrently with *N. oostreum* in *C. virginica* (Sprague 1949, Lauckner 1983). The definitive host for *N. prytherchi* is the stone crab *Menippe mercenaria* (Sprague 1949) which were also found at the Caloosahatchee sites sampled (Volety et al. 2003a). Reports concerning the pathogenicity of *Nematopsis* infections in *C. virginica* are inconclusive but most researchers have found no evidence to indicate the parasite is harmful to oysters (Overstreet 1978, Lauckner 1983).

The cnidarians observed infesting the gills of Caloosahatchee oysters did not seem to cause any pathologic distress to the hosts, even though large numbers of the symbiont were present in many of the collected animals. Several species of warm water hydrozoan cnidarians inhabit body folds of bivalve molluscs as inquiline symbionts. Of these, only one North American genus, *Eutima*, is found in *C. virginica* attached to the oysters gills, mantle and palps (Mulholland & Fredl 1996, Samler 2001). Those researchers collected oysters from numerous sites along the southern Atlantic and Gulf coasts and observed hydroids only in Florida from Tampa Bay and St. Augustine southward (Samler 2001). Thus, it is probable that the hydroids observed in the Caloosahatchee oysters belong in this group. The light to heavy infestations with no observable pathology detected is not unusual because prior studies of these hydrozoans reported that oysters can tolerate thousands of polyps without apparent harm (Samler 2001). Infestations appeared to have a seasonality because hydroids at those collection sites with the heaviest prevalence were observed in spring and early summer months (February to June) whereas almost none were detected in midsummer through fall. The summer decline was similar to other closely related bivalve-inhabiting cnidarians, *Eugymnanthea inquilina*, observed in mussels and clams from the Ionian Sea, Italy (Piraino et al. 1994) and Japanese waters (Kubota 1983). Kubota (1983) reported a summer decline in hydroids from Japanese waters noting that polyps became very small, and often degenerated, at the end of medusoid production. In addition, Samler (2001) reported hydroid numbers in Florida waters declined rapidly following large drops in temperature or salinity after heavy summer rains. Experiments determined critical temperatures and salinities for these hydroids to be between 10 and 33°C and 8‰ to 34‰ respectively (Samler 2001). A drop in salinity occurred in the Caloosahatchee study sites during the summer months of the study. Salinities, which averaged 31‰ during most of the study, dropped to under 10‰ at most sites and to less than 5‰ for weeks at each occurrence, especially at Cattle Dock and those collection sites close to it, where the hydroids were most prevalent (Volety et al. 2003a, Volety et al. 2003b).

The turbellarians found infesting the gills of Caloosahatchee

oysters elicited a light to moderate hemocytic response from the gills and formed a mucus coat around themselves. However, since the prevalence was low (1% to 4%) and the numbers of individuals infesting individual oysters were few, the worms are probably more commensal than parasitic. Turbellarians are predominantly free-living predators or scavengers but certain members of the order Alloeocoels are intimately associated with marine molluscs (Lauckner 1983). Alloeocoel turbellarians have been reported in the mantle cavity and on the gills of numerous bivalves in Europe, Japan, North America, and Mexico (Lauckner 1983, Fleming 1986, Brun et al. 1999, Aguirre-Macedo & Kennedy 1999). In the Gulf of Mexico, members of the genus *Urastoma* ("oyster gill worm") have been observed infesting the gills of *C. virginica* from Tabasco State, Mexico (Aguirre-Macedo & Kennedy 1999) and the Mississippi coast (Overstreet, personal communication). These organisms have been reported most frequently as commensals in bivalves, causing very little pathology and no mortalities (Robledo et al. 1994). Brun et al. (1999) have confirmed the turbellarian has a definite attraction to oyster mucus and shows a greater preference for oysters over *M. edulis* and *M. arenaria* in Atlantic Canada. This lends credence to the hypothesis of Fleming (1986) that *U. cyprinae* is attracted to and probably feeds on mucus produced by the oyster's gills.

Previous researchers have suggested that the copious mucous secretions produced by the worms forms a cocoon-like envelope to protect it from unfavorable environmental conditions or the negative effects of the oysters' defensive mechanisms (Bataller et al. 1999, Bataller et al. 2003). The fact that mucus surrounding the helminths, in this survey, was full of oyster hemocytes supports a defensive role for this response because mucous secretions by these worms may immobilize or make it more difficult for oyster hemocytes to attack them. It is well known that mucous production in animals plays an important role in defense mechanisms. For example, mucous secretion in marine fish gills is their chief defense against gill parasites (Khan 1987, Lafferty 1997).

Although the worms did not appear to cause significant pathology in *C. virginica*, studies with mussels, *Mytilus galloprovincialis*, from Spain have shown that *Urastoma cyprinae* causes serious disruption of gill filaments and significant hemocytic infiltration in the affected areas where the worms were found. This physiologic effect combined with large numbers of the flatworm was believed to significantly reduce feeding capacity in infested hosts (Robledo et al. 1994, Brun et al. 1999).

Encysted digenetic trematode metacercaria found infesting the gonoducts of oysters, beyond taking up space and eliciting localized hemocytic responses to necrotic worms, caused no significant damage to the host. These worms belong to a large group of trematodes collectively known as echinostomes. Digenetic trematodes in the family Echinostomatidae use gastropod and bivalve molluscs as intermediate hosts and have a worldwide distribution (Lauckner 1983). Metacercarial stages of this worm have been reported in *C. virginica* along the Gulf Coast by Little et al. (1966, 1969), Winstead et al. (1998) and Fisher et al. (2000). This group of trematodes is recognizable by the presence of a collar of spines around the oral sucker (Cheng 1973). Little et al. (1966) reported the occurrence of encysted metacercariae of another echinostome, *Acanthoparyphium spinulosum*, in the mantle of up to 100% of *C. virginica* collected near Port Isabel, Texas and on the eastern Gulf coast; 90% of oysters from one Tampa Bay, Florida site were reported to harbor encysted metacercariae of an *Echinostoma* sp. in their gonoducts (Winstead et al. 1998, Fisher et al. 2000). The

encysted worms observed in the Caloosahatchee oysters are probably in the genus *Echinostoma* also, because they have the same number of collar spines and were found in the gonadal follicles just like those reported from Tampa Bay. However, unlike the trematodes reported from Texas, the worms from Florida could not be identified to species because infected oyster tissue fed to chicks and ducklings did not develop into fully mature adults (Overstreet, pers. comm.). The metacercariae found in Florida and Texas oysters caused little observable tissue reaction. Even though cercariae of this group have penetration glands and have been reported encysted in the tissues of other marine molluscs (Stunkard 1937, Cheng et al. 1966) they did not penetrate the tissues of the Caloosahatchee oysters. Instead, the trematode only infested the gonadal follicles that are, in essence, an extension of the outer environment. As such, the oysters did not have tissue "infections" and probably did not respond to the parasite because the follicle epithelium was not breached or damaged. It is also possible that the cercariae were induced to encyst before penetrating tissue by the oyster's humoral defense mechanisms. Cheng et al. (1966) reported that sera from oysters *C. virginica* and *C. gigas* could immobilize cercariae of another echinostome, *Himasthla quissetensis*, before they could penetrate their tissues. However, the oysters do respond to dead or dying worms with massive hemocytic reactions (Winstead et al. 1998, Fisher et al. 2000). Oysters, infested with parasites, kept in laboratory aquaria for 2 mo in Texas (Little et al. 1969) and 1 y in Florida (Winstead et al. 1998) contained up to 70% living encysted trematodes, which may indicate the oysters did not detect the worms presence and therefore did not respond. The common life cycle of these parasites involve birds, humans, and other mammals as definitive hosts and a gastropod snail as the first intermediate host. Free swimming cercariae from the snail penetrate and encyst in various bivalves or fish. These second intermediate hosts are then consumed by the definitive host to complete the life cycle (Cheng 1973). There did not appear to be any seasonality of infestation because oysters from Tarpon Bay were observed to have the parasite all months of the year.

Another trematode, *Proctoeces* sp., infesting the gonoducts of the Caloosahatchee oysters were unencysted, caused no pathology, occurred at low prevalences, and were associated with attached hooked mussels *Ischadium recurvum* (Volety, pers. comm.). Unencysted metacercariae of the fellodistomatid trematode *Proctoeces maculatus* have been reported in bivalves from Europe and the Atlantic coast of North America (Stunkard & Uzzmann 1959, Lauckner 1983). In the Gulf of Mexico the worm has been reported in the gonadal follicles of *C. virginica* from Pensacola Bay, Florida; Mobile Bay, Alabama; and Pascagoula Bay, Mississippi (Winstead & Couch 1981, Couch 1985). The occurrence of unencysted trematode metacercariae in these oysters indicates the worms may be "progenetic" or precocious last-larval stages. The term "progenetic" is applied to sexually mature, ovigerous digenetic trematodes occurring in invertebrates (Aitken-Ander & Levin 1985) which, instead of using a definitive vertebrate host, can abbreviate its life cycle by maturing and completing its life cycle in a single molluscan host. This characteristic of the genus *Proctoeces* (i.e., to be in different stages within a mussel) is well documented and discussed by Cheng (1967). The presence of attached hooked mussels on the Caloosahatchee oysters is significant because sporocysts, cercariae and, progenetic stages of *Proctoeces* have been found in hooked mussels from Louisiana (Hopkins 1954a), Mississippi (Overstreet, pers. comm.), and Texas

estuaries (Wardle 1980). It is possible that the attached mussels on the Caloosahatchee oysters are the source of the trematode and the worm exits the mussel as a cercariae, becoming available to either a definitive fish host or another mussel, and contacting neither, enters the oysters genital orifice by chance. From there it migrates to a gonadal follicle or duct and may either mature and complete its life cycle using the bivalves as surrogate hosts or remain in a metacercariae stage awaiting a mollusc-eating fish (Sparidae or Labridae). It is pertinent to note that pinfish, *Lagodon rhomboides*, and sheepshead, *Archosargus probatocephalus*, were both collected in the vicinity of sampling sites (Volety et al. 2003a). Seasonality of infestation could not be determined due to the low prevalence (<1%) of the trematode.

Branching sporocysts from the trematode *Bucephalus* sp. invaded vesicular connective tissue throughout the oyster and in heavy infections completely destroyed or "castrated" the gonads via mechanical damage. Digenetic trematode infections by the genus *Bucephalus* or "bucephalosis" in *C. virginica* is known to extend from Rhode Island to Texas, but is not continuous throughout this range (Hopkins 1954b, Sparks 1985). *Bucephalus* infections in Gulf coast oysters have been reported from Louisiana estuaries (Hopkins 1954a, Hopkins 1957, Menzel & Hopkins 1955, Turner 1985, Gauthier et al. 1990), Pensacola Bay, Florida (Winstead, personal observation); Apalachicola Bay, Florida (Menzel et al. 1969); and Tampa Bay, Florida (Fisher et al. 2000). Pathology of *Bucephalus* infections in the Caloosahatchee oysters is identical to that described in earlier studies. Trematode sporocysts invade the gonad and, via mechanical pressure, totally disrupt its integrity. Any remaining gametes become degenerate and reduced in size, more by metabolic rather than mechanical damage, resulting in parasitic castration (Sparks 1985). In heavy infections growing sporocysts invade the connective tissue surrounding the digestive diverticula, obliterating the space they normally occupy. Later, sporocysts replace connective tissue in the mantle and invade water tubes of gill lamellae (Sparks 1985). Hemocytic response from the oyster is minimal except when sporocysts become degenerate or die. The end result is almost total replacement of oyster tissue by sporocysts, which in late stage infections probably lead to the death of the oyster, especially in adverse environmental conditions (Hopkins 1957, Sparks 1985). Except for some Louisiana bayous in which Hopkins reported 10% to 25% of oysters with *Bucephalus* infections, all the above studies reported low prevalence of infections (<2%) and the Caloosahatchee oysters were no exception with most sites around 1% and one with 3%. It is pertinent to note that prevalence of *Bucephalus* infections has been reported to be highest in low salinity and lowest in open bays with higher salinity (Hopkins 1957, Sparks 1985). This seems to be the case with the Caloosahatchee oysters; all sites with the infection had lower salinity conditions and higher salinity sites had no infections. This correlation of bucephalosis with lower salinity is probably due to the presumed definitive and second intermediate hosts (the true gar, *Lepisosteus* and mullet, *Mugil cephalus*) preference for lower salinity waters. There did not appear to be a seasonality of infection for any sites where the parasite was found.

Larval cestodes, *Tylocephalum* sp., infected oysters at high prevalences at most stations and caused a severe host response consisting of fibrous encapsulation and hemocytic infiltration to resorb the parasite. Host response was in accordance with the observations of Lauckner (1983) and Sparks (1985). Many of the Caloosahatchee oysters had multiple infections of the parasite. Of

60 infected *C. virginica* inspected by Cake and Menzel (1980), some had as many as 125 worms in their tissues but none were weak or moribund or showed any loss of body weight (Lauckner 1983). The location of encapsulated worms varied but in general they were found in loose connective tissue between the digestive diverticula and the stomach and less frequently in the connective tissue of the gills and mantle. The most common route of infection is through the stomach epithelium and there is no appreciable host response until the cestode gets into the vesicular connective tissue around the stomach, mantle or in the gills (Lauckner 1983). After the parasite gets through the stomach epithelium, connective tissue fibers begin to form around it and capsule formation begins. The end result is a thick intercellular cyst of fibrous material between the vesicular connective tissue and a hemocytic infiltration (Lauckner 1983). Encapsulated worms are gradually resorbed by oyster hemocytes, which probably means that *C. virginica* is not a totally compatible host for *Tylocephalum* (Cheng 1967). The resorption of *Tylocephalum* metacystode larvae has also been reported in other bivalves with the exception of the pearl oyster *Pinctada* spp., which is considered to be the normal intermediate host for *Tylocephalum* sp. (Lauckner 1983).

Tylocephalum sp. acutate glando-proceroids or metacystodes are common parasites in marine mollusks throughout the world (Cake & Menzel 1980, Lauckner 1983, Sparks 1985) and have been reported in *C. virginica* from numerous sites along the eastern Gulf of Mexico (Sparks 1963, Cake 1977, Cake & Menzel 1980, Couch 1985, Fisher et al. 1996, Fisher et al. 2000). Cake and Menzel (1980) reported metacystodes in oysters sampled from 22 of 27 sites between Bay St. Louis, Mississippi and Everglades City, Florida. They reported an average prevalence of 43.5% (range 0% to 100%) infection, which is similar to what other researchers have observed in Gulf coast oysters. Also, it was not unusual to find 100% of oysters infected with the parasite at some sites. The Caloosahatchee oysters also had metacystode infections at all sites and some, especially the higher salinity sites at Tarpon Bay and Greys Reef, had prevalences of up to 90%.

The fact that many individual oysters at the Tarpon Bay and Greg's Reef sites harbored multiple species of parasites indicates a fairly rich diversity of organisms in those areas compared with the other sites. It is pertinent that those sites, where fewer parasites were observed, were closer to habitat that has been severely altered by human activity (freshwater release from Lake Okeechobee, pollution and shoreline disruption). Habitat alterations can lead to decreases in parasitism of a population by eliminating the intermediate and/or definitive hosts the parasites need to complete their life cycles (MacKenzie et al. 1995, Lafferty 1997, Lafferty & Kuris 1999). For example, Burn (1980) observed reduced diversity of species and parasite numbers in fish from Raritan Bay, which was heavily impacted by human activity, compared with Block Island Sound which was less polluted. Snails, *Cerithidea californica*, separated from a marsh by a parking lot and highway were completely uninfected with larval trematodes compared with a 25% prevalence in snails inhabiting the adjacent marsh. The definitive hosts for these worms and birds, were absent in the cut off marsh area because of the habitat alteration (Lafferty 1997). Thus, a paucity or abundance of parasites, especially helminths, in a target population, in this case oysters, may be an indirect indicator of the biotic diversity in that area. If a host population harbors a large number and variety of parasites, especially metazoan parasites, it is probable that a rich assortment of free living fauna is living in or moving through that area (Cheng 1973). It is important

to note, however, that there are many instances where total numbers of the same species of parasite may increase due to human activities, such as pollution (MacKenzie et al. 1995, Lafferty 1997). For example, increased Trichodinid gill ciliates in fish seems to be correlated with a toxicant's ability to impair mucus production, which is a fishes chief defense against those parasites (Khan 1987, MacKenzie et al. 1995). Therefore, as Lafferty (1997) states "it is important to take into account the relative sensitivities of each group and have a through understanding of the associations

between the wide range of parasites and the impacts upon them" before one correlates anthropogenic impacts with biotic responses.

ACKNOWLEDGMENTS

We thank Ms. Sharon Thurston, and Erin Rasnake for providing field assistance and Lee Courtney for providing help with figures. The authors also thank Dr. Robin Overstreet for his critical review of the manuscript.

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A REVIEW OF THE MARKET STRUCTURE OF THE LOUISIANA OYSTER INDUSTRY: A MICROCOSM OF THE UNITED STATES OYSTER INDUSTRY

FERDINAND F. WIRTH* AND TARA M. MINTON

Food and Resource Economics Department, Indian River Research and Education Center,
University of Florida, IFAS, 2199 South Rock Road, Ft. Pierce, Florida 34945

ABSTRACT Louisiana enjoys the highest annual oyster production among all states, contributing over thirty percent of the total domestic supply of the eastern oyster, *Crassostrea virginica*. Over the last 2 decades, changes in oyster habitat, concerns over shellfish safety and government regulations have seriously affected the oyster industry in Louisiana and the United States. In an attempt to fill a research void identified by the Gulf Oyster Industry Program, this analysis identifies and assesses the Louisiana oyster industry size and value, product lines, market structure, distribution, and distribution channels. Recommendations to the Louisiana oyster industry include offering an array of value-added products that fit today's busy lifestyle, and addressing safety concerns through quality assurance to restore consumer confidence.

KEY WORDS: oyster, distribution, Louisiana, market structure, value-added products

INTRODUCTION

The eastern oyster, *Crassostrea virginica*, also known as the American oyster, is the principal oyster species being cultivated in the Gulf of Mexico and along the Atlantic coast. In the early 1800s, Louisiana's first commercial oyster operations began in the estuaries near the present Mississippi River Delta. In the mid-1800s, immigrant fishermen realized that high-quality oysters could be produced by transferring small "seed" oysters from the natural reefs near the delta to bedding grounds closer to the Gulf of Mexico. These higher salinity areas did not support substantial natural populations due to high predation, but were excellent for fattening and growth of transplanted seed (Perret & Chatry 1988).

By the turn of the 20th century, Louisiana's oyster-producing areas had been divided into public seed grounds (most productive natural reefs) and private bedding grounds. Public reefs are opened each year in the fall and winter both for collection of seed oysters and harvesting of market oysters. Much of the remaining oyster-producing areas were made available for private leasing. Thus, the basic organization of the Louisiana oyster fishery was established. The state supplied the seed oysters, and the private leaseholders would transfer the seed to their leases for growth to market size. This growout of transplanted seed is a type of oyster production known as on-bottom or extensive culture and is the typical method of farming oysters in the United States today (Supan 2002).

In many ways, the Louisiana oyster industry can be viewed as a microcosm of the entire United States oyster industry, with comparable production and marketing challenges and opportunities. In the last decade, changes in oyster habitat, production technology, concerns over shellfish safety, and government regulations have seriously affected the oyster industry in Louisiana and throughout the United States. High production costs are restricting expansion by established culturists and serving as barriers to entry into the industry. Land for shore-side facilities is in demand by housing, recreational, and manufacturing companies, which can pay premium prices to obtain choice sites. Many coastal activities and competing uses for areas suitable for oyster culture are not compatible with oyster growout procedures.

Determining market characteristics including sale, distribution, and preferred product forms was identified as a major research need by the Gulf Oyster Industry Program (Supan 2000). This report was constructed in an effort to fill this research void, and reports the results of a review of the Louisiana oyster industry's market structure. The analysis identifies and assesses industry size and value, product lines, market structure, distribution, and distribution channels. Key facets of the Louisiana oyster industry are also compared and contrasted with other major oyster-producing states.

INDUSTRY SIZE AND VALUE

World oyster production has increased 10-fold in the last 50 years. In 1950, the United States accounted for 80% of the world oyster supply. By 2000, the United States market share had fallen to only 6%, although the United States landings have remained relatively constant during that time. Figure 1 shows the United States and world oyster landings from 1950 to 2000. The oyster market is currently dominated by China, which supplied 77% of the world's oysters in 2000 (Food and Agricultural Organization 2002).

In 2001, the United States oyster landings totaled 14,800 metric tons (32.7 million pounds) of meat at a value of \$80.9 million (National Marine Fisheries Service 2002a). Figure 2 depicts the United States oyster landings and dockside prices, both nominal and real (in 1996 dollars) from 1974 to 2001 (US Department of Labor 2003). Oyster landings remained relatively steady during this time period; values generally increased until about 1994, after which they tended to decrease. Because nominal prices seem to have increased dramatically across the period, real prices have remained stable. There is a significant negative correlation between the United States commercial oyster landings and real dockside value (Pearson correlation coefficient = -0.418 , $P < 0.05$).

Louisiana currently enjoys the highest annual oyster production, by weight, among all states in the nation, with annual landings generally in the 4,500–5,900 metric ton (10–13 million pound) range. Figure 3 shows eastern oyster landings in Louisiana from 1950 to 2001. As the current production leader, Louisiana contributes significantly to the Gulf and United States supply. During the 5-year period from 1997 to 2001, Louisiana contributed 54% of the total oyster landings in the Gulf Region. At the national level, Louisiana averaged 32% of the total landings during the

This research was supported by the Florida Agricultural Experiment Station, and approved for publication as Journal Series No. R-09928.

*Corresponding author. E-mail: ffwirth@ifas.ufl.edu

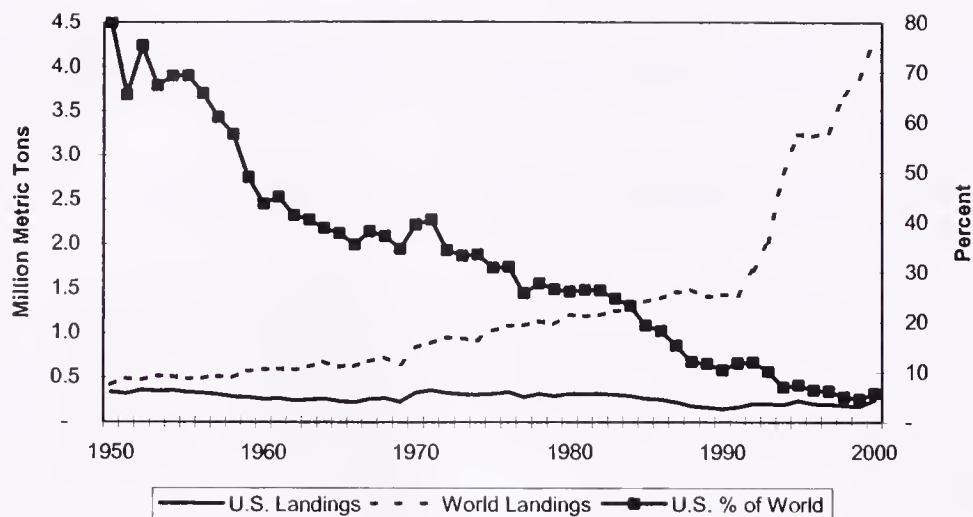


Figure 1. United States and World Oyster Landings, 1950–2000.

same 5-year period. In 2001, Louisiana provided 37% of the United States' oyster production with 6,000 metric tons (13.2 million pounds) of meat (Louisiana Department of Wildlife and Fisheries 2002). Over 1,100 Louisiana producers used 170,000 hectares (420,000 acres) to produce over 77,000 metric tons (170 million pounds or 1.7 million sacks) of oysters in 2001 (Louisiana Cooperative Extension Service 2002). In February 2003, there were over 8,500 active oyster leases in Louisiana covering more than 166,000 hectares (409,000 acres) of leased water bottom (Louisiana Department of Wildlife and Fisheries 2003).

The value of Louisiana's oyster industry has been trending upward. Averaging just over \$6.0 million annually during 1961 to 1966, the Louisiana oyster industry reported a farm value of \$20.7 million in 2001 (Louisiana Cooperative Extension Service 2002). Although some of the increase was due to inflation, much of the increase in value resulted from an increase in production and price. The significant, and continuing, decline in the United States and, especially, Chesapeake Bay oyster production since 1981 may have expanded demand for Louisiana oysters. This decline in the United States production is believed to have had a positive influence on Louisiana dockside oyster prices. However, there is evidence that the demand for oysters dramatically declined during the late 1980s and early 1990s, possibly as a result of consumer concerns over product contamination, health, and nutrition (Lipton & Kirkley 1994).

PRODUCT LINES AND PROCESSING

Product Lines

The number of different types of oyster products being produced throughout the United States from the eastern oyster, *Crassostrea virginica*, has declined from 15 unique products in the 1970s to only 6 products in 1991. Products include counter stock half-shell oysters (unprocessed), fresh raw shucked oysters, breaded oysters, canned stews, canned oysters and smoked oysters (Lipton & Kirkley 1994). Oysters currently produced in Louisiana fall under 2 product classifications: raw shucked, and counter stock sold in the shell. Production is largely raw shucked oysters. Counter stock oysters are those of the highest grade, the most carefully cultivated that are served on the half-shell at restaurants and oyster counters.

Value-added Processing

In the United States, processed eastern oysters are almost exclusively fresh, raw product. From 1970 to 1990, the percentage of oysters processed as fresh, raw shucked product increased from 76% to 92%. The only other significant processed oyster product is breaded oysters, which are available fresh or frozen, either raw or cooked. Breaded oyster production fell by one-half from 1970 to 1990, down to less than 726 metric tons (1.6 million pounds).

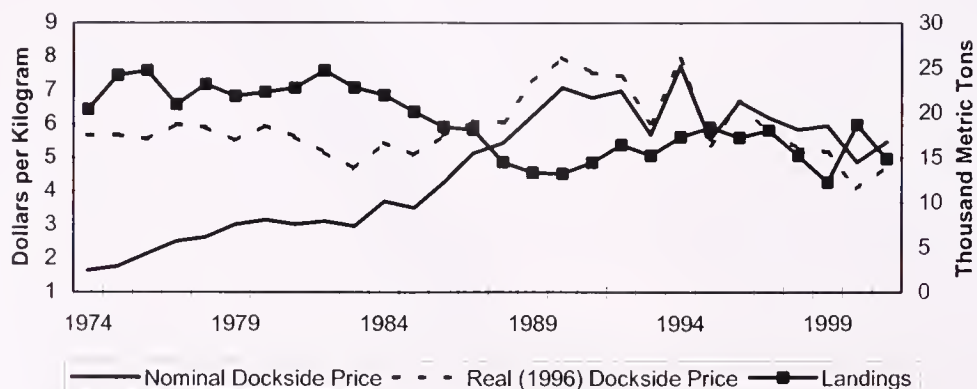


Figure 2. United States Oyster Landings and Nominal and Real Dockside Values, 1974 to 2001.

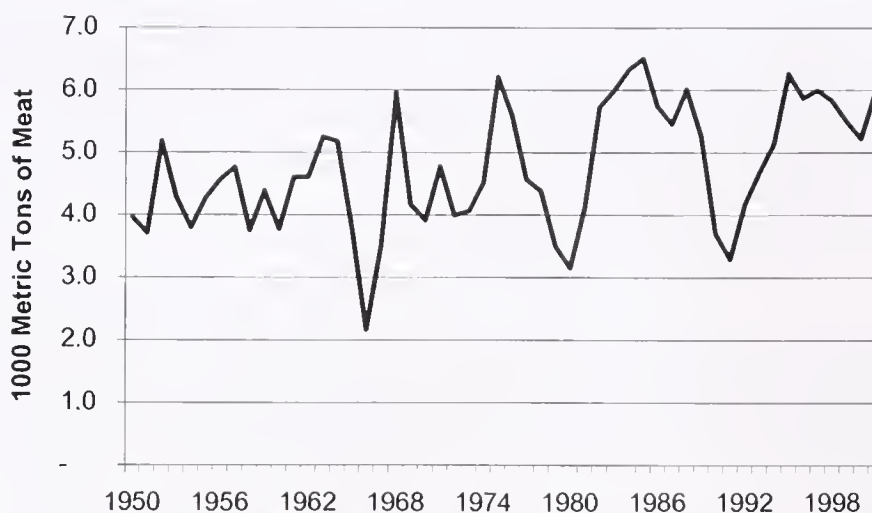


Figure 3. Eastern Oyster Landings in Louisiana, 1950 to 2001.

Production of canned oysters and oyster stews from eastern oysters had virtually disappeared by 1990. Stews are increasingly being made with imported oysters. Only one firm reported processing smoked eastern oysters in 1990 (Lipton & Kirkley 1994).

Because Louisiana accounts for about half the annual Gulf region oyster harvest, oyster processing activities in the state are generally well below 50% of the Gulf region total and appear to be declining. Louisiana's share of the Gulf region oyster processing activity has fallen from 38.5% by value during 1971 to 1975 to only 28% by value during the 1981 to 1985 period. No further value-added processing activities beyond shucking, such as bread-ing, were conducted by the 30 Louisiana first buyers interviewed by Keithly and Roberts (1988). An examination of the Thomas Food Industry Register (1995 edition) identified few oyster processors in Louisiana. Only nine companies processed fresh, canned, or frozen oysters. Three companies were involved with private label manufacturing. No Louisiana companies were identified as producing breaded oysters, stew, or chowder.

Keithly and Roberts (1988) offered 3 possible explanations for Louisiana's small and declining share of Gulf region oyster processing activities. First, much of Louisiana's harvest leaves the state to be processed in other Gulf states. Second, "though Louisiana oyster companies do conduct primary processing activities (i.e., the shucking of oysters), they perform very little additional value-added services, such as the bread-ing of oysters." Finally, the declining share could reflect the demise of the Louisiana oyster canning business, which had disappeared by the early 1980s, a victim of canned oyster import competition from South Korea and Japan.

Retail Consumer Products

Five supermarkets in Baton Rouge, Louisiana were surveyed in October 1997, during the oyster season, to identify value-added processed oyster products readily available to consumers. Four of the stores represented large supermarket chains that had extensive fresh and frozen seafood departments, and offered a wide assortment of value-added seafood products. The fifth supermarket was a locally owned, independent supermarket. Oysters were available in 4 product forms: fresh shucked, canned whole, canned smoked, and canned oyster stew. Five Louisiana brands of fresh, refrigerated shucked oysters, packaged ungraded in several different sizes

of plastic containers, were readily available in 4 of the 5 stores. One store offered a brand of Washington State farm-raised fresh oysters. Four brands of canned whole oysters were identified, 2 from California companies, 1 from a New Jersey company, and 1 from a Louisiana company. However, the canned oysters from 3 brands, including the Louisiana brand, were products of Korea. A similar situation exists with smoked oysters, which were available from 6 companies. The products from 5 of the 6 companies, including the Louisiana company, originated in Korea. Only 2 brands of canned oyster stew were identified. One of the stews contained Pacific oysters, whereas the source of the oysters in the other stew was not identified.

The absence of local, further processed oyster products in Louisiana supermarkets mirrors the situation in the Mid-Atlantic states. In March 1999, during the Mid-Atlantic oyster season, three supermarkets in the Newark, Delaware area were inspected to identify local and value-added oyster products. Two of the supermarkets are national chains, and the third is part of a large regional supermarket chain. The results were virtually identical to the situation in the Louisiana supermarkets. The fresh, shucked oysters were harvested locally from surrounding Mid-Atlantic states. No other locally produced value-added oyster products were identified. All value-added oyster products, primarily whole canned and smoked canned oysters, were products of Korea or China. Lipton (1999) confirmed the lack of local value-added oyster products in the Mid-Atlantic region. "Oysters are either being sold in the shell or as raw shucked oysters. I haven't seen any local oysters in the value-added market for years."

DISTRIBUTION CHANNELS

Market Segments

The market for shucked and half-shell Louisiana oysters is primarily domestic, located in coastal states, and segmented geographically by region. Only three Louisiana companies export oysters outside of the United States. The primary market is Louisiana and the surrounding Gulf states. Additional strong markets exist in the Mid-Atlantic states and the West Coast, to fill excess demand created by declining oyster stocks in those regions.

Dealer Numbers and Behavior

As of May 2003, the Interstate Certified Shellfish Shippers List reported 50 shellstock shippers, 48 shucker-packers, 18 reshippers, and 2 repackers as certified dealers in the Louisiana shellfish industry (Interstate Shellfish Sanitation Conference 2003). According to the National Marine Fisheries Service Seafood Processor's Survey, the largest four oyster processors in Louisiana represented over 70% of the state's shucked oyster market in 2000 whereas the top 10 processors represented over 91% of the market (Isaacs 2003).

Keithly and Roberts (1988) surveyed 30 primary dealers to document procurement and marketing activities in the Louisiana oyster industry during the 1985 to 1986 season (September 1985 to August 1986). Figure 4 provides an overview of oyster procurement and product form distribution for the 30 dealers. The dealers reported obtaining nearly 55,000 metric tons (120 million pounds or 1.2 million sacks²) of oysters from independent oystermen or their own leases. The dealers also secured another 11,000 metric tons (24.7 million pounds or 247,000 sacks) from out of state, primarily Texas, and another 10,600 metric tons (23.3 million pounds or 233,000 sacks) from other Louisiana dealers. The dealers had a total of 77,000 metric tons (170 million pounds or 1.7 million sacks) for processing and/or sale. The majority of the total (60%) was resold as sacked oysters. A small amount (7%) was boxed. The final third were shucked and marketed in various sized containers.

Thirty-five percent of the sacked and boxed oysters were directed to in-state markets, whereas the other 65% was directed to out-of-state markets. The majority of these out-of-state shipments were sacked oysters, which were primarily destined for processing plants in Alabama, Mississippi, and Florida. The survey results indicated that 30% to 35% of Louisiana's 1985 to 1986 harvest

was processed out-of-state. Of the shucked product, 58% was directed toward out-of-state markets, while 42% was sold in-state. An unknown amount of the in-state sales may have left Louisiana through secondary outlets and been subject to additional value-added services.

OYSTER MARKET STRUCTURE

Consumption

According to data from the National Marine Fisheries Service report, Fisheries of the United States, and authors' calculations, total US consumption of oysters was over 26,000 metric tons (58 million pounds) and per capita consumption was 0.09 kg (0.20 pounds) of meat in 2001. Figure 5 depicts the US per capita consumption of oysters from 1971 to 2001. Per capita consumption was highest in 1972 at 0.19 kg (0.42 pounds) of meat, and lowest in 1999 and 2001 at 0.09 kg (0.20 pounds) of meat (National Marine Fisheries Service 2002a). Limited evidence cited by Lipton and Kirkley (1994) suggests that the decline in demand for oysters is a result of health/nutrition concerns, product safety, water pollution, economic fraud (adulterated product), and media publicity.

There has been extensive publicity about dangers of consuming shellfish, particularly raw molluscan shellfish. In July 2003, a Louisiana court found a restaurant and the State Department of Health liable for the death of a person who ate raw oysters containing *Vibrio vulnificus* (Florida Department of Agriculture and Consumer Services 2003a). On May 1, 2003, the state of California imposed an emergency regulation limiting importation of summer harvested Gulf oysters to "only those oysters subjected to post harvest treatments that reduce *Vibrio vulnificus* to non-detectable levels will be allowed during April through October" (Florida Department of Agriculture and Consumer Services 2003b). Florida, Louisiana, and Mississippi have challenged this rule, stating that it violates the policies of the Interstate Shellfish Sanitation Conference.

Two post harvest treatments designed to combat *Vibrio vulnificus* and *Vibrio parahaemolyticus* bacteria are currently in use in the Louisiana oyster industry. Ameripure Processing Company, Inc. uses a warm water bath followed by an ice-cold shock bath to reduce the existence of pathogens to undetected levels in raw oysters (Ameripure 2004). No chemicals or irradiation are used. Oysters are sold raw in the shell and must be shucked fresh. The process also reduces aerobic bacteria, which slows the natural rate of spoilage, extending the shelf life of the product (Ameripure 2004).

Motivatit Seafoods, Inc. uses a high-pressure processing technology, Fresher Under Pressure developed by Avure Technologies, Inc. (Motivatit Seafoods Inc. 2004). The oysters are exposed to 45,000 pounds per square inch of pressure for 2 minutes. Much like the Ameripure process, high-pressure treatments reduce harmful bacteria to nondetectable levels and decrease spoilage microorganisms. In addition, when placed under pressure, oysters release their adductor muscle from the valve freeing them from the shell, essentially shucking themselves (Food Engineering 1999). No heat is used in this process.

Determinants of Consumer Behavior

In a nationwide study, Cheng and Capps (1988) found that own price elasticity and household size were generally the key factors in explaining the variation of household expenditures on oysters for at-home consumption. The study measured own-price elasticity of demand, which describes the relationship between price and

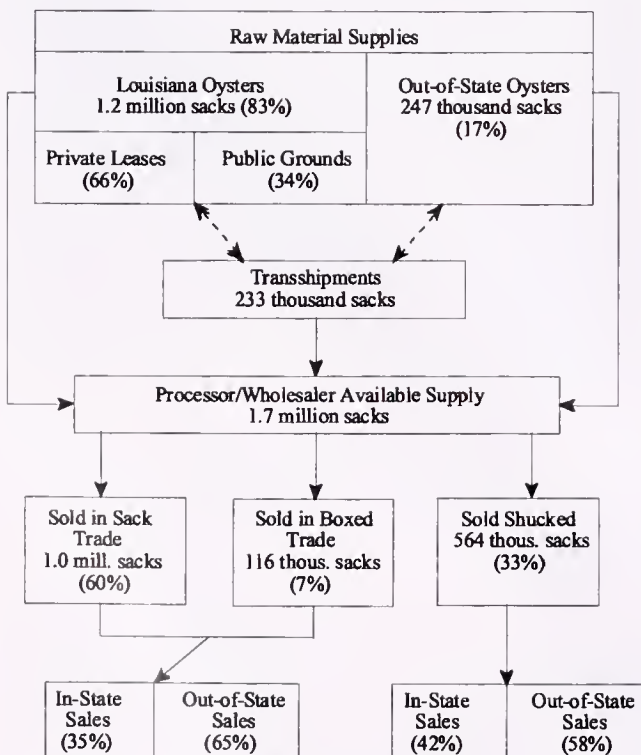


Figure 4. Louisiana Wholesalers/Dealers Oyster Procurement and Distribution Source: Keithly and Roberts, 1988 A sack of Louisiana oysters is 0.045 metric tons or 100 pounds (Keithly 2003).

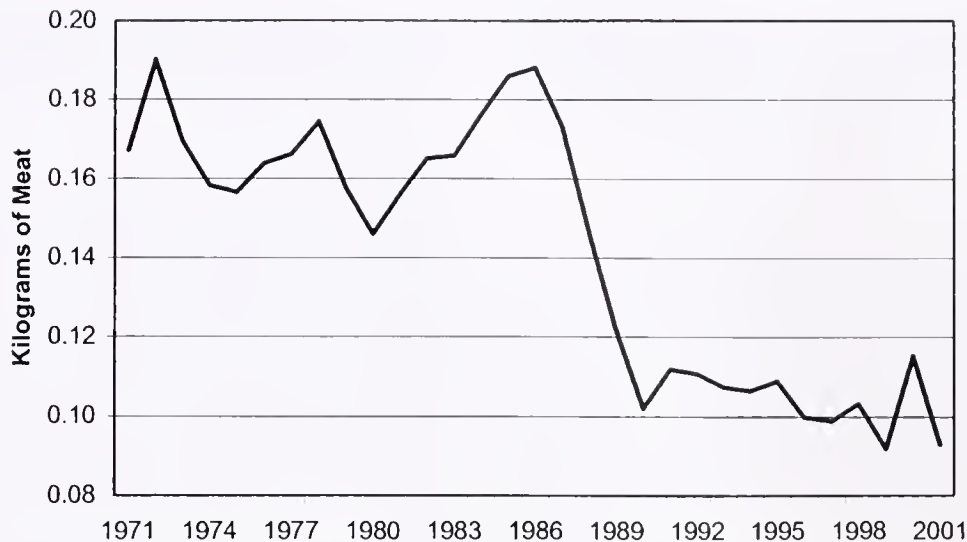


Figure 5. United States Per Capita Consumption of Oysters, 1971 to 2001.

quantity demanded of a good. They found that own-price elasticity for oysters was statistically significant and had a value of -1.1320 . This means that a 1% increase in the price of oysters would decrease the quantity of oysters demanded by -1.1320% (Penson et al. 2002). The Cheng and Capps' study also measured income elasticity of demand, which describes the changes in quantity demanded of a good based on changes in income. They found household income elasticity of oysters was statistically significant and had a positive value of 0.1769 , indicating oysters to be a normal good. For normal goods, in this case oysters, large increases in income are required to generate substantial increases in consumption (Penson et al. 2002).

Lin and Milon (1993) used a double-hurdle model to investigate whether the decision to consume oysters (participation decision) has determinants different from the frequency of consumption (consumption decision). A rating scale was used to measure perceptions of 5 food attributes commonly cited as the basis for food choices: safety, taste, nutritional value, freshness, and cost. Taste perceptions were highly significant determinants of oyster participation and frequency decisions. Nutrition perceptions were positive and significant in frequency decisions only. Cost perceptions negatively influenced oyster consumption frequency but did not affect oyster participation. Thus, the decision whether to consume oysters was primarily determined by taste perceptions. Once that decision was made, taste, nutrition, and cost perceptions determined consumption frequency. Of the personal characteristic variables, age, gender, and race were significant in the frequency decision for oysters, but only education was significant for oyster participation, with more educated persons being more likely to consume oysters. A similar study by House et al. (2003) found that concern for product safety did not significantly influence whether participants consumed oysters, but did significantly influence the decision of how frequently oysters were consumed. Twenty-five percent of consumers indicated they ate oysters less frequently due to product safety concerns.

Capps and Lambregts (1991) used scanner data to study the demand for fish and shellfish (shrimp, crab, lobster, oysters, and scallops) products in a Texas retail food firm predominantly catering to high-income customers. Own-price elasticity and seasonality were not statistically significant factors in purchases of oys-

ters. Demand for oysters was affected by advertising. Own-advertisement elasticity (0.059) was positive. Cross-advertisement elasticities for finfish (-0.224) and poultry (0.473) suggested that advertisement space for finfish and poultry influences oyster purchases.

Little is known about the at-home demand for specific forms of oyster products (i.e. canned and stews), demand for away-from-home consumption of oysters, or changes in oyster demand over time. Lipton and Kirkley (1994) evaluated the trends in at-home oyster consumption, comparing data from the 1977/1978 and the 1987/1988 USDA National Food Consumption Survey. They found that the share of oyster purchases represented by each of 3 oyster product types (fresh/frozen shucked oysters, canned smoked or salted oysters, and oyster stew) had changed over the 10-year period. The share of fresh/frozen-shucked oyster had decreased only slightly, from 54% to 50%. The share of canned oysters doubled, from 25% to 50%. The share of oyster stew fell from 21% to 0%. The increasing share of canned oysters, combined with a high price for smoked eastern oysters compared with the Pacific oyster counterpart, led Lipton and Kirkley (1994) to conclude that canned, smoked eastern oysters is one product for which "there appears to be wide open market" for oyster producers.

"The changing socio-demographic and economic structure of the US population, as well as changes in consumer lifestyles, has contributed to the increased popularity of food away from home." Nayga and Capps (1995) measured the effect of socioeconomic factors on the probability of consuming fish and shellfish away from home. The findings generally indicated that older people, people with higher income, people with smaller household sizes, and people on a special diet are more likely to eat fish and shellfish away from home. The study recommended that fish marketers and processors emphasize these factors when formulating marketing strategies designed to promote consumption.

Complements and Substitutes

According to Cheng and Capps (1988), the cross-price elasticity (0.1991) between red meat and oysters suggested that red meat and oysters were gross substitutes. Cross-price elasticity between oysters and poultry was not statistically significant.

Capps and Lambregts (1991) found that, in Texas, cross-price effects for other shellfish (1.21) are positive and statistically significant, supporting the notion that other shellfish species (shrimp, crabs, lobster, and scallops) are substitutes for oysters. Cross-price effects for finfish, pork, poultry, and beef were not significantly different from zero.

Domestic Competitors

The major competitor for Louisiana oysters are oysters produced in other regions of the United States. Oysters are harvested in 18 of the 21 contiguous coastal states. Four species of oysters are presently being cultivated in the United States. *Crassostrea virginica*, the eastern oyster, is the principal species on the Atlantic Coast and the Gulf of Mexico, and accounts for most US oyster landings. *Crassostrea gigas*, the Pacific oyster, is the primary culture species on the West Coast and second in volume produced, with approximately 3,600 metric tons (8 million pounds) produced annually (National Marine Fisheries Service 2003). Two species of the genus *Ostrea* are also cultivated at low levels. *Ostrea edulis*, the European oyster, is grown in Maine, and *Ostrea lurida*, the native West Coast Olympia oyster, is the basis for a small industry in the states of Washington and Oregon (Burrell 1985).

The catastrophic decline in natural eastern oyster populations in the Chesapeake Bay left the Maryland and Virginia oyster industries in near collapse. Because the main cause of the historical decline is most likely overharvesting, Dermo and MSX, occurrences caused by parasitic protozoa, have essentially wiped out remaining natural stocks and made traditional culture methods in the region unproductive. Figure 6 shows eastern oyster harvests in Maryland, Virginia, and Louisiana from 1950 to 2001. In Virginia, oyster production peaked in 1958 with 11,800 metric tons (26 million pounds) and has declined drastically ever since, reporting just over 91 metric tons (0.2 million pounds) in 2001. In Maryland, the harvest peaked in 1954 and again in 1973 with 9,000 metric tons (20 million pounds). The Maryland industry produced 590 metric tons (1.3 million pounds) in 2001 (National Marine Fisheries Service 2003).

Other species of bivalves, including clams, mussels, and scallops also compete with Louisiana oysters for market share. The main non-oyster bivalve competitor is the hard clam, *Mercenaria mercenaria*, which is widely distributed throughout the Atlantic and Gulf of Mexico coastal waters, and has long supported a major commercial fishery in the New England and Mid-Atlantic coastal

states. US cultured clam output was 4,900 metric tons (10.7 million pounds) in 1999, with a reported value of \$42.0 million. On the top ten US seafood list for the last decade, per capita consumption of clams was 0.22 kg (0.49 pounds) in 2000 (Johnson 2001).

Imports and Exports

The United States imports, largely from the Asian region, are principally low-value canned or smoked oysters. Imports were high in 1987 at 23,600 metric tons (52.0 million pounds), however they declined to 12,500 metric tons (27.5 million pounds) by 1990 (Lipton et al. 1992). In 2002, US oyster imports totaled 8,700 metric tons (19.1 million pounds) worth \$36.9 million (Economic Research Service 2003). Imports of fresh and frozen oysters accounted for approximately one-third of total oyster imports in 2002, whereas canned oysters accounted for the remaining two-thirds imported for consumption (National Marine Fisheries Service 2002b). The United States exports far fewer oysters than are imported. Oyster exports in 2002 totaled 1,800 metric tons (3.9 million pounds) with a value of \$8.7 million (Economic Research Service 2003). The United States oyster exports are comprised of fresh and frozen product; no canned oysters were exported by the United States in 2002 (National Marine Fisheries Service 2002b). Because little of the United States oyster harvest is canned, the degree of competition between imports and Louisiana oysters is debatable. In a survey of United States oyster wholesalers by Lipton and Kirkley (1994), 44% of the Louisiana dealers believed imports posed a problem for increasing domestic sales.

CONCLUSION

This analysis has identified and explored several key issues that the Louisiana oyster industry must confront and solve to assure future industry growth and success. There seems to be room for expansion in the United States and Louisiana oyster-processing sector, especially value-added services such as breaded or smoked oysters. There are now basically only 2 eastern oyster products, whole oysters and fresh-shucked oysters. As oyster production increases through industry revitalization efforts and aquaculture production, new product forms will have to be developed (Lipton & Kirkley 1994). Expansion of the processing sector will generate increased oyster industry revenues through time. Other value-added shellfish products (especially shrimp and clams) have been created for the institutional trade and as convenience items for home use. Specialty products such as soups, stews, and breaded

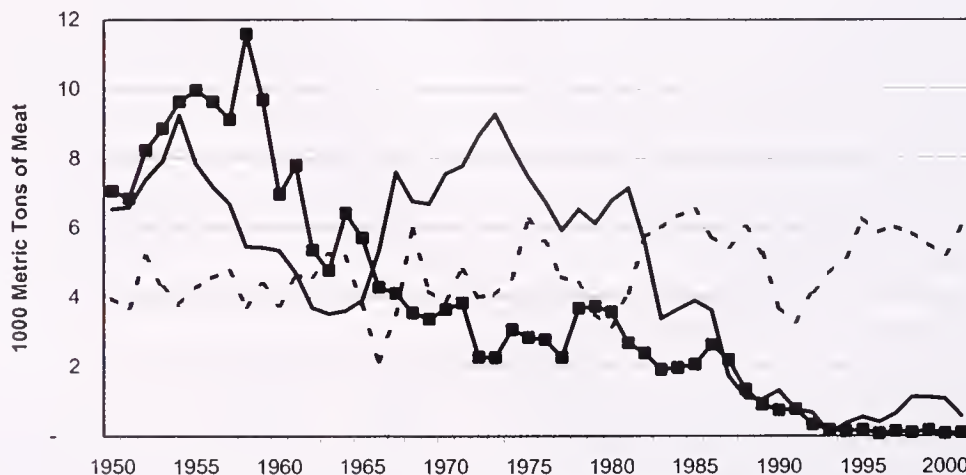


Figure 6. Eastern Oyster Landings for Selected States, 1950 to 2001.

oysters prepared for institutional service would take advantage of the increase in the number of people eating out. Consumer preference studies may provide direction for product development (Supan 2000).

Oyster processors wishing to exploit value-added product opportunities should follow the example of Tallmadge Brothers Inc. of Connecticut, which added to its product line of oysters, clams and shucked meats by investing in a blast freezer and introducing individually quick frozen half-shell oysters and clams on vacuum pack trays. Tallmadge's Marketing Director provided the rationale for the new product introduction by stating that "to penetrate the retail sector we are going to have to find a way to make clams and oysters accessible to today's busy two-income family" (Rheault 1997).

Finally, efforts to combat the market impacts of shellfish contamination, through harvesting regulations and innovations in post harvest treatments, must continue. Studies of consumer attitudes

toward different oyster quality assurance processes may shed light on the relative market merits of the different techniques. Oyster quality assurance may very well be the marketing key to restoring consumer confidence in Louisiana oysters.

ACKNOWLEDGMENTS

The authors thank Steve Koplin, Fishery Reporting Specialist with the National Marine Fisheries Service; Walter Keithly, Associate Professor at Louisiana State University; Patrick Banks, Martin Bourgeois and Jack Isaacs, Marine Fisheries Division of the Louisiana Department of Wildlife and Fisheries; and Kathy Davis, Coordinator of Economic Analysis at the University of Florida for their assistance in producing this report. The authors also thank Chuck Adams, Peter Stoffella, Suzanne Thornsby, Al Wysocki, and two anonymous reviewers for their helpful comments.

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*Real dockside value (nominal prices adjusted for inflation) was calculated in real 1996 dollars based on Producer Price Index, Unprocessed and Packaged Fish and Shellfish, US Department of Labor (Series #WPU0223).

*A sack of Louisiana oysters is 0.045 metric tons or 100 pounds (Keithly 2003).

REPRODUCTIVE BIOLOGY OF THE “COPEY” SNAIL *MELONGENA MELONGENA* (LINNAEUS, 1758) IN CISPATÁ BAY ON THE CARIBBEAN COAST OF COLOMBIA

SANDRA HERNÁNDEZ¹ AND WOLFGANG B STOTZ^{2*}

¹Fundación Humedales, Calle 97 No 21-42 Bogotá, Colombia

²Universidad Católica del Norte, Casilla 117 Coquimbo, Chile, wstotz@ucn.cl

ABSTRACT The reproductive cycle of the “Copey” snail *Melongena melongena* at Cispatá Bay was examined from October 1998 to September 1999. Gonad maturation was studied through macroscopic and microscopic observations on the gonads of females >70 mm (total shell height), showing that individuals were mature throughout the year, but with a period of maximal maturity in March which coincided with the time of maximum recruitment. The mean size at first maturity was 52 mm for males and 65 mm for females. A small number of intersex individuals (4%) (masculinized females, with a normally developed ovary and a rudimentary penis; feminized males with a normally developed testis and gonopore) occurred at intermediate sizes. The proportion of males in the population decreases with the increase in individual size, with only females present at the biggest sampled size-class (mean size of 98.5 mm shell height), suggesting protandric hermaphroditism. If this is proven to be true, artisanal fishing preferring larger animals, could severely interfere in the success of reproduction.

KEYWORDS: Reproductive cycle, *Melongena melongena*, Caribbean, protandric hermaphroditism, intersex.

INTRODUCTION

The “Copey” snail *Melongena melongena* (Linnaeus, 1758) is widely distributed in the Gulf of México, the Caribbean and the Antilles. The snail lives on the shallow areas of coastal lagoons, mangroves and estuaries, being an important component of the fauna on soft or muddy bottoms. The snail tends to concentrate on areas with high densities of its prey, which are bivalves, other gastropods, ascidians and carrion (Hathaway & Woodburn 1961, Hawkins 1973, Rodríguez 1976, Flores 1980, Cosel 1986, Morton 1986, Dalby 1989, Villareal 1989, Bowling 1994). Considering this behavior, fishers sometimes use mangrove roots, covered with the mitilid *Mytilopsis sollar* as bait, letting the snails aggregate on the bait from one day to the next. The method of collection of these snails includes walking over the bottom and locating the snails beneath the mud with the foot. By doing this, the fishers are not able to discriminate size, all sizes being fished. *M. melongena* attains sizes up to 200 mm of shell height (Clench & Turner 1956, Abbott 1974, Flores 1980, Díaz & Puyana, 1994). In Cispatá Bay individuals between 12 and 151 mm shell height are found, the mean size of capture being 57 mm.

M. melongena copulates from December to July in Colombia (Rodríguez 1976) and then produces egg capsules. Egg masses of *M. melongena* have a common basal layer. The number of capsules produced fluctuates between 27–31, located along a string which measures up to 218 mm in length. Some modified, sterile egg capsules are produced and serve to anchor the egg mass on the soft bottom. This sterile capsules are smaller than fertile capsules, but have the same shape and are spaced approximately 28 mm apart at one end of the string. The average distance between fertile capsules along the string is only 6 mm, this distance getting shorter towards the last capsules produced. Egg masses are mostly buried in soft bottoms close to the vegetation on the coast. The capsules contain at least 185 to 290 embryos according to D’Asaro (1997), or 300 to 400 embryos according to Flores (1980), each embryo measuring 250 to 300 μ m. The development within the capsules takes 20 to 25 days. By day 16 a well developed veliger larvae is observed, having four large velar lobes with a double row of strong

cilia on their outer edges. The foot and operculum are well developed, the larvae apparently being able to crawl. By day 25 or older, when removed from the capsule, the larvae no longer protruded the velar lobes, but they crawl actively and could rapidly turn over the shell with the aid of the foot. When the capsules open, most of the young snails, now incapable of swimming, immediately crawl and appeared to be feeding (Clench and Turner 1956)

Rodríguez (1976) reported that the smallest mature females found in Cartagena Bay measured 80 mm and the males 65 mm of shell height. Besides this observations by Rodríguez, information on the gonad maturation of *M. melongena* in Colombia is scarce.

With few exceptions, prosobranch gastropods are gonochoric, typically with internal fertilization, although some species demonstrate protandric hermaphroditism (Gallardo 1989). Rodríguez (1976) identified *M. melongena* females with shell heights between 40 and 62 mm having an incipient development of the penis. Species related to *M. melongena*, such as *Busycon carica* and *M. corona*, have similar characteristics (Castagna & Kraeuter 1994, Zetina 1999).

Previous observations have suggested that in nursery habitats, small individuals were mostly males and in areas where growth and maturation occur, larger individuals were predominantly females (Hathaway & Woodburn 1961, Woodbury 1986, Zetina 1999). Intersex individuals and sex dominance in different size categories, suggest that *M. melongena* may be a consecutive protandric hermaphrodite. This requires verification, since artisanal fishing activities, which prefer larger sizes, may negatively affect the population by seriously altering sex proportions. The fishery for *M. melongena* occurs throughout the Caribbean, but as the snail is only used for local consumption, it does not appear in the statistics. Nevertheless, the fishery is important, for example Hernández (2001) estimating for the Cispatá Bay area (the bay and nearby sloughs) that 70% or the total biomass of the snail population is fished, representing for local fishers ca. 30% of their total income. The meat of the snail is mainly used around the Caribbean to offer appetizers (the popular “botanas”) in beach restaurants of tourist areas.

The objective of the present study was to describe the reproductive cycle and pattern of recruitment, sex ratio and the presence of intersex individuals in *M. melongena* in Cispatá Bay. This in-

*Corresponding author: wstotz@uch.cl

formation should provide a base for fisheries resource administrators to design biologically sound management measures for this resource.

MATERIALS AND METHODS

Study Area

Individuals of *M. melongena* were collected from October 1998 to September 1999 from the Cispatá Bay area (includes the nearby sloughs and the area close to Amaya Beach) (Fig. 1).

Reproductive Cycle

Each month from October 1998 to September 1999, snails were searched for in the field (as fishers do, with the foot), collected manually and measured. This sampling was part of a study regarding distribution, abundance and growth of the snail. Thus, a great number of individuals (over 100), representing the entire depth distribution, was sampled. For the study of the reproductive cycle the largest 30 females, but measuring at least 70 mm of shell height (females of that size were observed to lay eggs), were selected. Due to the fishery, in which the average size of capture is around 50 mm, with few individuals reaching 80 mm or more, large individuals are scarce in the field, so when ever possible, larger individuals were bought from fishers. To get the 30 females, collected individuals had to be sacrificed to verify sex. As the aim of the study was to know the time of reproduction of *M. melongena* in Cispatá Bay, the analysis was concentrated on females, considering that only when they are mature, copulation can occur. As perhaps not all females, but probably all males, may reproduce each year (as described for other gastropods), this method may sub estimate the number of reproducing individuals within the population. But the general seasonal pattern will not be affected by this.

The tissues of each measured individual (total shell height in mm), were removed, and the gonad separated from them. Each component was then weighed separately (drained wet) to the nearest 0.01 g. The gonads were fixed in Bouin's fluid and prepared using traditional histologic procedures to obtain thin sections (6 μ m) stained with Ehrlich's Haematoxylin and eosin. Three sections were taken from each embedding block, each 360 μ m apart to avoid including the same reproductive follicles in the different sections.

Two methods were used to establish the reproductive cycle. The first included a macroscopic quantitative evaluation (gonad index = GI) and the second was based on microscopic qualitative evaluation (index of gonad maturity = IGM).

The gonad index (GI) is represented by:

$$GI = (\text{weight of the gonad} / \text{total weight of tissues}) \times 100$$

For the total weight of tissues all soft tissues of the animal, including head, foot with operculum, visceral mass, mantle, as well as the gonad, were included.

The index of gonad maturity (IGM) characteristics were established for each stage of gonad development, as defined by Ramorino (1975):

- Evacuated (I): walls of acini with a corrugated appearance and abundant phagocytes within them.
- Maturing (II): internal walls of the acini with pedunculated and pyriform vitellogenic oocytes and the presence of a few phagocytes and large spaces between them.
- Maximum maturity (III): the walls of the acini take on a po-

lygonal form without spaces between them and the presence of abundant nutritive cells.

IGM was calculated using the following equation:

$$IGM = (\sum (n \cdot F) / N) \times 100$$

Where F = stage of gametic development (I–III), n = number of individuals in stage F and N = number of total individuals.

Recruitment

Using the size frequencies obtained throughout one sampling year and the growth parameters calculated by Hernández (2001), the FISAT program routine for recruitment patterns (Gayanilo & Pauly 1997) was used. This permits determination of the number of recruitment pulses per year and their intensities. For this study, recruitment was defined as the addition of new individuals into the benthic population.

Mean size at first maturity

Based on information reported by Rodríguez (1976) and Flores (1980), which describe that *M. melongena* copulates from December to June, the egg masses being most abundant in February and March, an intensive sampling was done between December 1998 and March 1999. Ten individuals were obtained for each 10 mm size class, beginning with 20 mm individuals. This number of individuals was decided, considering the possibility to really obtaining them in the field, or being able to buy them from fishers. The lower limit of 20 mm corresponds to the smallest individuals fishers include in their catch. Both males and females were identified, using histologic sections of the gonads. Individuals were rated as either immature (abundant connective tissue; absence of, or very few acini), or mature (Stages I–III). The data for each sex were fitted to a logistic model using the CurveExpert 1.3 program (Copyright (c) 1995–1997 Daniel Hyams.) based on the equation:

$$Y = a / (1 + b e^{-cx})$$

Where Y = Relative accumulated frequency (%), a = constant, b = slope, c = constant and x = total shell height.

The mean size at first maturity was estimated at a level of 50% of the relative accumulated frequency (Zetina 1999).

Sex ratio and Intersex individuals

Through macroscopic and microscopic observations, individuals were identified as either males or females and thereby an estimate of the sex ratio was obtained. When sex determination between macroscopic and microscopic examination did not agree, individuals were classified as in the intersex phase based on criteria used by Reed (1993) and Zetina (1999). At the macroscopic level, masculinized females present an atrophied penis which was smaller than that of normal males. An ovary was observed at the microscopic level. The feminized male had a gonopore with no macroscopic differences from that of a normal female, and microscopically they have a testis.

RESULTS

Cispatá Bay (Fig. 1) is located in the Department of Córdoba, on the Caribbean coast of Colombia, between 9°26' and 9°21'N and 75°54' and 75°45'W. The bay is located south of the Gulf of Morrosquillo. It has an irregular topography, is bordered by mangroves, and has estuarine waters with a mean depth of about 2 m.

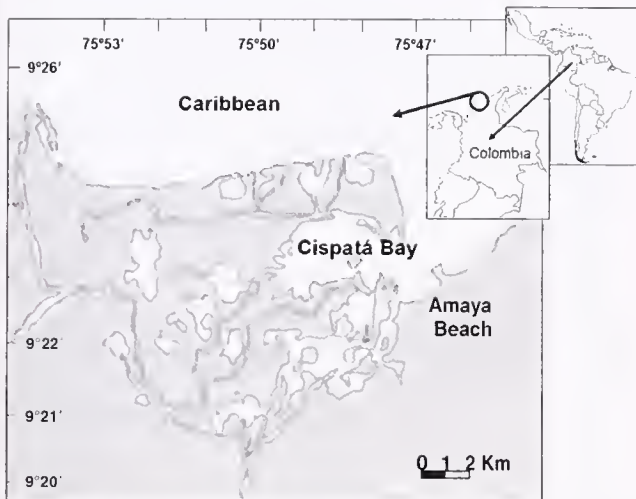


Figure 1. Map of Cispatá Bay and related inner and outer (coastal) sloughs on the Caribbean coast of Colombia.

(Sánchez *et al.* 1997). Inland from the bay occur 12 sloughs which connect with it by channels (Fig. 1).

The study area experiences four climatic seasons including, (a) the dry season from January through March, (b) transition to the rainy season from April through August, (c) the rainy season from September through November and (d) transition to the dry season in December. These seasons are affected by the Caribbean Current which flows from east to west and by the Darien Countercurrent, or Panama Current, which flows northward along the coast (Ramírez 1994). The tides are semidiurnal, with an amplitude of less than 1 m; the annual mean temperature is 26.7°C and the annual precipitation is between 900 and 1200 mm (Patiño & Flores 1993).

Reproductive cycle

A high proportion (>50%) of mature individuals (Stage III) were observed from January through September, except for the months of February, April, and June in which the proportion diminished (Fig. 2). Each peak of mature individuals was preceded and followed by maturing individuals (Stage II). Spawned individuals (Stage I) appeared almost throughout the year, with the exception of January and the period of August and September. Thus, spawning appears to occur between February and July, and

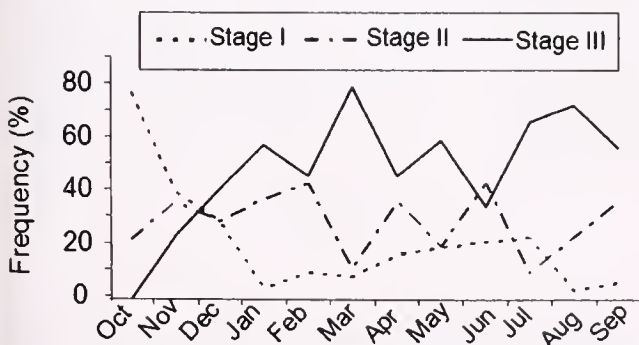


Figure 2. Variation in the Gonad Maturity Stages (see text) of *Melongena melongena* females in Cispatá Bay.

then again in October, the month in which the greatest proportion of spent individuals appeared.

The index of gonad maturity (IGM) increased from October to December, staying high for the rest of the year, but with smaller peaks in January, March, and August (Fig. 3). The gonad index (GI) showed March, June, and September as months with maximum gonad development (Fig. 4). Main reductions in the gonad index, suggesting spawning, were observed during October–November and March–April, and a smaller one in June (Fig. 4).

Recruitment

The recruitment pattern, estimated using the growth parameters $L_{\infty} = 163$ mm and $K = 0.2 \text{ y}^{-1}$ estimated by Hernández (2001), shows that individuals entered the population throughout the year with a maximum in March (Fig. 5). This coincided with the period of maximum maturity, according to the proportion of individuals in Stage III and highest IGM (Figs. 2 and 3). Recruitment throughout the year coincides with the observation that mature individuals occur for the entire year, existing no defined seasonal pattern of reproduction. A small peak was observed in March.

Mean size at first maturity

According to Figure 6a, 50% of males reached sexual maturity at 52 mm shell height; such results fit the logistic model. It was thus estimated that all males were capable of reproduction at a size of 73 mm (Fig. 6a). Data for females also fitted the logistic model. Females became sexually mature shortly after the males at a shell height of 65 mm, with all females capable of reproduction at 77 mm (Fig. 6b). Spawned females 74–75 mm in height were identified in April.

Sex ratio

The sex ratio changed with age (Fig. 7). In the smallest size class (26.5 mm) females predominated, but only 8 individuals of that size class were examined. In the second analyzed size class (32.5 mm) males predominated and then tended to decline toward greater size classes (32.5–92.5 mm). In the greatest analyzed size class (98.5 mm) only females were found, but again, it was only a small sample (4 individuals) (Fig. 7).

Intersex individuals

Few individuals (13) could be classified as in the intersex phase, representing only 4% of the 325 individual examined. Individuals with both male and female characteristics had both a

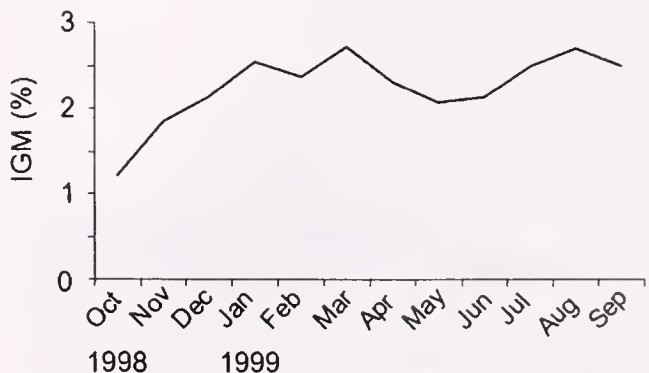


Figure 3. Variation in the Index of Gonad Maturity (IGM) of *Melongena melongena* in Cispatá Bay.

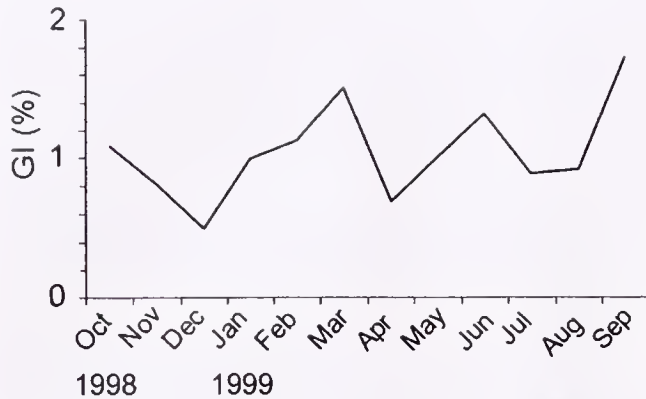


Figure 4. Variation in the Gonad Index (GI) for female *Melongena melongena* ≥ 70 mm in shell height in Cispotá Bay.

gonopore and an incipient penis similar to normal males, although microscopic examination confirmed they were females (masculinized females) with normal developed ovaries. These individuals measured 40–70 mm in shell height (Table I). Microscopic observations confirmed that some individuals with a gonopore were males (feminized males), and these were 55 to 73 mm in shell height presenting a normal developed testis. All these individuals were classified as in the intersex phase.

DISCUSSION

The coincidence between the major recruitment pulse and the maximum maturity, described by various methods (stages of maturity, IGM, GI) with also coincident results, suggest that the reproductive cycle described is reliable. While the peak of maturity occurs in the dry season, the presence of mature individuals throughout the year shows that variation between dry and rainy season seems to affect little, supporting a hypothesis of continuous reproduction and recruitment, a condition common in neotropical gastropods (Weber 1977). Rodríguez (1976) reported that in another area of the Caribbean, copulation by *Melongena melongena* occurred between December and July. These observations may have been biased by the methodology used by Rodríguez as they were based only on field observations where sampling days did not necessarily coincide with days when ovicapsules were present.

Continuous spawning, beginning at early age, represents a favorable condition for *M. melongena*, which is subjected to an artisanal fishery. According to growth data (Hernández 2001), *M. melongena* becomes sexually mature in its third year (at sizes

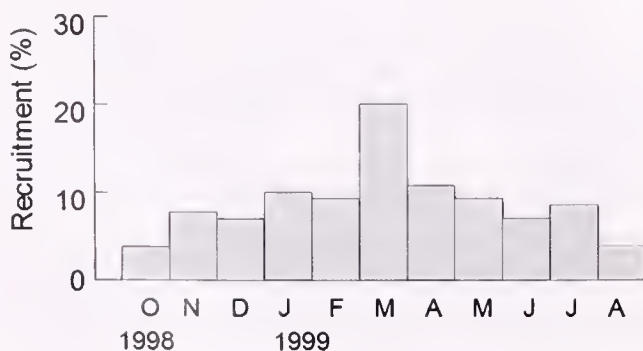


Figure 5. Temporal pattern of recruitment into the population of *Melongena melongena* in Cispotá Bay and related sloughs.

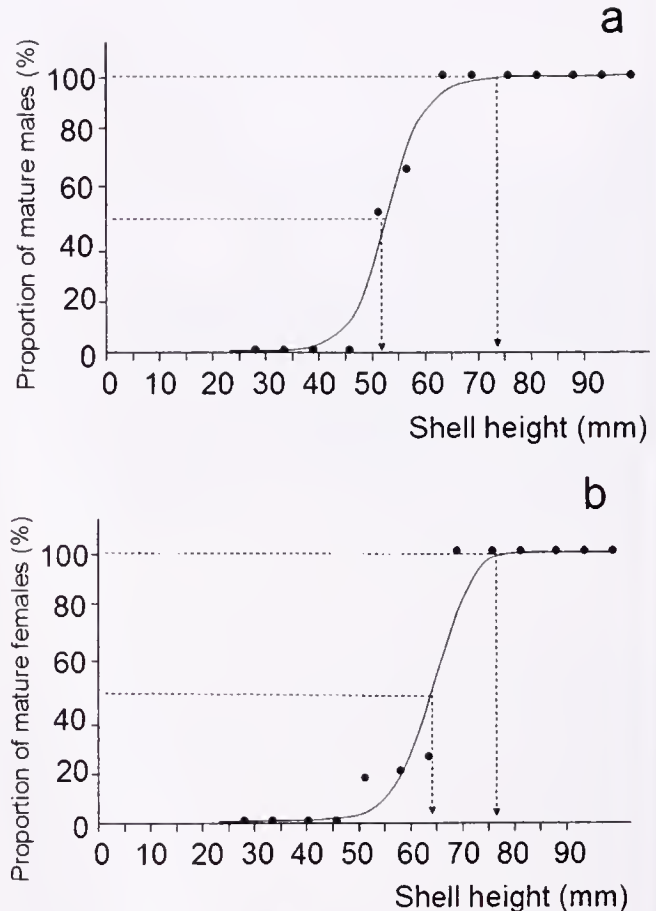


Figure 6. Sexual maturity of *Melongena melongena* in relation to size (total shell height) for (a), males and (b), females in Cispotá Bay.

between 52 and 65 mm). Nevertheless, the fishery is capturing individuals as small as 12 mm shell height, the main captures occurring from >40 mm, with an average size of 57 mm (Hernández 2001). Thus, most of the fished snails have not reproduced prior to capture. This is a situation which needs to be revised and regulated to prevent overfishing of this resource, af-

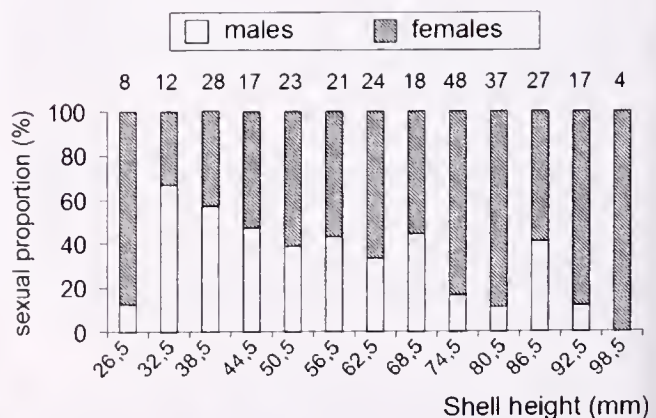


Figure 7. Sex ratio (%) of *Melongena melongena* in relation to size in Cispotá Bay. The x-axis shows the mean total shell height of each size class (mm). Numbers above columns represent the total number of individuals analyzed.

TABLE 1.

Intersex individuals identified for each size class interval of *Melongena melongena*.

Size class interval (mm)	Feminized males	Masculinized females
40		1
43		
46		1
49		2
52		
55	2	3
58	1	
61		
64		
67		
70	1	1
73	1	
TOTAL	5	8

fecting recruitment and thus population renewal. That is unless the fishery is inefficient, and a great proportion of individuals of all sizes escape capture.

As *M. melongena* appears to be gonochoric, the observation of intersex individuals generates doubts. The presence of such individuals confirms the observations of Rodríguez (1976), which, in agreement with the present study, reported masculinized females as occurring at sizes between 40 to 62 mm in shell height. For comparison, both feminized males and masculinized females were found in Cispata Bay, at a shell height range of between 40 and 73 mm.

The decrease in the number of males with increasing size suggests the occurrence of consecutive hermaphroditism. Dominance by females is however frequent in populations of gonochoric molluscs, and becomes accentuated with increasing age, eventually generated by differential mortality (Fretter & Graham 1962, Gibbs *et al.* 1987). It has been found in populations of *Nucella lapillus*, affected by imposex, that the males are more abundant than females, as the latter suffer heavy mortality (Gibbs & Bryan, 1986 and Gibbs *et al.* 1987). But the identification of intersex individuals also points to the occurrence of hermaphroditism. This may however, be due to other specific causes such as those cited by

Reed (1993) who indicated that sexual alternation in *Strombus gigas* could be due to abnormalities in the sex chromosomes. Horiuguchi *et al.* (1994) established that in neogastropods the development of male sex organs in females (termed "imposex") was a broad-scale problem described for numerous species. Gibbs *et al.* (1987) established that the presence of masculinized females in *Nassarius obsoletus* and *Nucella lapillus* could be produced by exposure of these species to toxic compounds present in antifouling paints such as tributylene and TBT (tributyltin chloride). Exposure to these pollutants of females of these species destroys the oviduct, suppresses oogenesis, and results in the development of a testis. The females become unable to reproduce and the population declines as a result (Gibbs & Bryan 1986). According to Horiuguchi *et al.* (1994), the degree of imposex in *Thais clavigera* and *T. bronni*, as indicated by the length of the penis, provides a rough estimate of TBT levels in the environment.

Event though, the change in sexual proportion with size and the existence of few intersex individuals suggests the occurrence of protandric hermaphroditism in *M. melongena*, the evidence is weak. If the occurrence of protandric hermaphroditism is true, however, the fishery for *M. melongena* may be interfering seriously with reproduction, as the individuals are not allowed to reach the larger sizes. This needs to be further studied, following over time the same individuals, to determine if changes in sexual proportion with size and intersex individuals are really evidence of protandric hermaphroditism, or result from other causes.

ACKNOWLEDGMENTS

We are grateful to Hanne Cogolio and Jesus Cantillo for help in field sampling, and to the snail collectors, especially "Tanque". We are also thankful for logistical support provided by Luz Marina Arias, Chief of the INPA office in Cordoba, and to Evila Jarava Ossa for her collaboration. Further thanks are extended to the personnel of the Amaya Research Station, to Marisol Romero of the U.Catolica del Norte, Coquimbo, for guidance in the histological interpretation of *M. melongena* gonads and to Piedad Victoria for review and edition of the Spanish text of this MS. We add final thanks to Adriana Zetina, Cristian Aycaguer and C. D'Asaro for sending reprints of their research which were important to our preparation of this MS. Two anonymous reviewers are acknowledged for their comments, which helped to improve the manuscript.

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TRANSPLANTING OF WILD AND CULTIVATED JUVENILES OF GREEN ABALONE (*HALIOTIS FULGENS* PHILIPPI 1845): GROWTH AND SURVIVAL

SERGIO A. GUZMÁN DEL PRÓO,^{1,3,*} JORGE CARRILLO-LAGUNA,^{1,3}
JORGE BELMAR-PÉREZ,¹ LAURA CARREÓN-PALAU¹ AND ALBERTO CASTRO²

¹Escuela Nacional de Ciencias Biológicas, I.P.N. Prol. Carpio y Plan de Ayala S/N, México, D.F. 11340; ²Sociedad Cooperativa de Producción Pesquera, "Bahía Tortugas" Bahía Tortugas, B.C.S.;

³Becario COFAA

ABSTRACT The growth and survival rate of wild and cultivated abalone juveniles (*Haliotis fulgens*) transplanted into the sea was measured from January 2000 to January 2003 in Bahía Tortugas, Baja California. A total of 306 wild individuals (17–90 mm shell length) and 529 cultivated juveniles (16–49 mm shell length), were tagged and released in the intertidal zone. Six surveys were carried out during the experiment. The recaptured organisms were released, each time, in the same place once the shell length was recorded. Growth data were fitted to Gompertz growth model. Mortality rate was estimated through an exponential decay model. The growth rate 1 year after release was 52.4 mm/year for wild abalone and 48.1 mm/year for cultivated abalone. In the third year these rates decreased to 30 and 28 mm/year respectively. No significant difference was found. Gompertz equation parameters were very similar for wild ($L_0 = 2.34$, $G = 4.30$; $g = 0.827$) and cultivated abalone ($L_0 = 2.14$, $G = 4.38$; $g = 0.812$). Natural mortality rates were $M = 1.20 \text{ y}^{-1}$ for cultivated juveniles and $M = 0.87 \text{ y}^{-1}$ for wild juveniles. No significant difference was found between both. Sighting efficiency rate was: 74% for both types of juveniles. This proportion is indicative that wild and cultivated juveniles had a similar dispersal and cryptic ability. We conclude that annual survival rates: 30% for cultivated juveniles and 42% for wild juveniles could be sub estimated.

KEY WORDS: growth, survival, transplanting, juveniles, Baja California, *Haliotis fulgens*

INTRODUCTION

Fishing of abalone (*Haliotis* spp.) is one of the leading economic activities in the regional economy of Baja California. Native populations of this mollusk have undergone a sharp decline in numbers since the 1970s, as a result of increased fishing pressure as well as possible climate changes (Guzmán del Prío 1992, 1994). Commercial landings and biomass are estimated to have declined in some areas to one-tenth of their original numbers, in comparison to the 1950s (Guzmán del Prío 1992, 1997).

The outplanting of hatchery-reared abalone for the purpose of restocking natural reefs has been practiced routinely in Japan (Kojima 1995, Seki & Tanaguchi 2002). Recently, successful seedings experiments have been conducted in Australia (Dixon et al. in press). These countries have also been interested in promoting the sale of cultivated abalone in international markets.

In Mexico, the fishing cooperatives of Baja California maintain abalone hatcheries for the specific purpose of restocking their reefs. Similarly, they occasionally transplant wild juveniles from "stunted stock" areas to banks known for their "good growth." To date, however, both practices have lacked the follow-up that might allow assessment of the survival of the seeded stock as well as of its impact on the total biomass of the wild populations. Although data exists on the growth of adult *Haliotis fulgens* Philippi 1845 and *Haliotis corrugata* Gray 1828 (Marín 1981, Turrubiates 1989, Shepherd et al. 1991, Guzmán del Prío & Lopez-Salas 1993), little is known of the juvenile stages of these species.

The purpose of this study is to determine the growth rate and survival of wild and cultivated abalone juveniles following their release at sea, to evaluate the impact of enhancement programs on wild populations of these mollusks.

STUDY AREA

The study was done in the rocky intertidal zone of "Varadero" at Clam Bay (27°37'11"N and 114°50'38"W), a small cove

located southeast of Bahía Tortugas (Fig. 1). The study site is a low-relief shelf with many imbricate rocks sheltered from the dominant northwest winds. Juveniles of *H. fulgens* are abundant in this location.

METHODS

A total of 835 juveniles of green abalone (*Haliotis fulgens*), 529 cultivated and 306 wild, were tagged. Cultivated juveniles ranged in size from 16 to 49 mm length, with a mean of 23.5 mm, and were 1 year old. Wild juveniles had a broader size range, from 17 to 90 mm (mean 53.5 mm), which is approximately equivalent to 1 to 2 years of age.

The wild specimens were captured at sites near Bahía Tortugas and taken to the laboratory in ice chests containing water and giant kelp (*Macrocystis pyrifera* (L.) Agardh 1820). They were measured and tagged along with the cultivated individuals that were obtained from the Aquaculture Unit of Cooperativa Bahía Tortugas. All specimens were allowed to rest for 24 hours in tanks with running seawater to cull damaged individuals.

Numbered polyethylene ribbon tags (Hallprint, South Australia) were fixed to the shells with cyanoacrylate glue (Three Bond TB-1747). Specimen length was measured to the nearest millimeter with vernier calipers.

A random mix of wild and cultivated juveniles was released at low tide in January 2000. In one case, the specimens were attached by hand to the underside or side of boulders, 30 × 60 cm on average, covered in the upper surface by calcareous or foliose algae, but with smooth undersides. Juveniles were planted at densities of 4 to 7 per boulder, according to the microhabitat observations of Carreón-Palau et al. (2003). In another case, 25 to 30 juveniles were placed in 30-cm long, 4-cm diameter PVC tubes in the laboratory, then taken to sea and deposited in cryptic locations among the rocks to allow the specimens to abandon the tubes gradually and choose suitable sites.

The release area was marked off by two 25-m long steel chains placed at right angles to the shoreline and separated from each

*Corresponding author. E-mail: sguzman@ipn.mx



Figure 1. Study area.

other by a distance of 75 m. Each chain was also marked at 5 m intervals with small plastic chains as reference points. The seeding and recapturing area was approximately 200 m².

Recaptures were undertaken in summer and winter months, six in total, from June 2000 to January 2003, recording the size and tag number of each specimen. Searching was done always on days when the tide was at its lowest, starting with the release sites and their surrounding area and applying a constant search effort of six people for 1 hour. However, because summer ebb tides (June to July) were not enough to leave the boulders totally exposed, and in addition, a heavy *Macrocystis* canopy is characteristic of this time of the year, the searching efficiency was seriously affected. Thus, we decided to use for the final analysis only the winter data surveys, when we found the lowest tides and less densities of macroalgae cover.

Most recaptured juveniles were measured *in situ*. When this was not practicable, the specimens were carefully detached to avoid excessive handling. After measurement, they were replaced on the rock, making sure they were attached to it.

The data of the mean length by age of recaptured specimens, both wild and cultivated, were fitted to Gompertz growth model (Rawlings 1988, Ricker 1975).

$$Lt = Lo * e^{G(1 - e^{-g * t})}$$

Where:

Lo = the initial SL at time t_0 .

G = the instantaneous growth rate at time t_0 , and

g = describes the rate of decrease of G .

Both, cultivated and wild mean length-increments, were compared by ANCOVA test (Scheffler 1980).

To estimate survival, the raw data for the total number of wild and cultivated specimens recaptured each year was corrected as follows: tagged specimens not sighted before the second and/or third recapture were considered to be survivors in the previous periods. This correction allowed us to obtain a more accurate estimate of minimum survival.

The proportion of individuals not sighted before, but sighted in

subsequent surveys was used to estimate the sighting efficiency (Dixon et al. in press). An Arcsine transformation (Krebs 1999) was used to normalize proportions prior to calculate sighting efficiency.

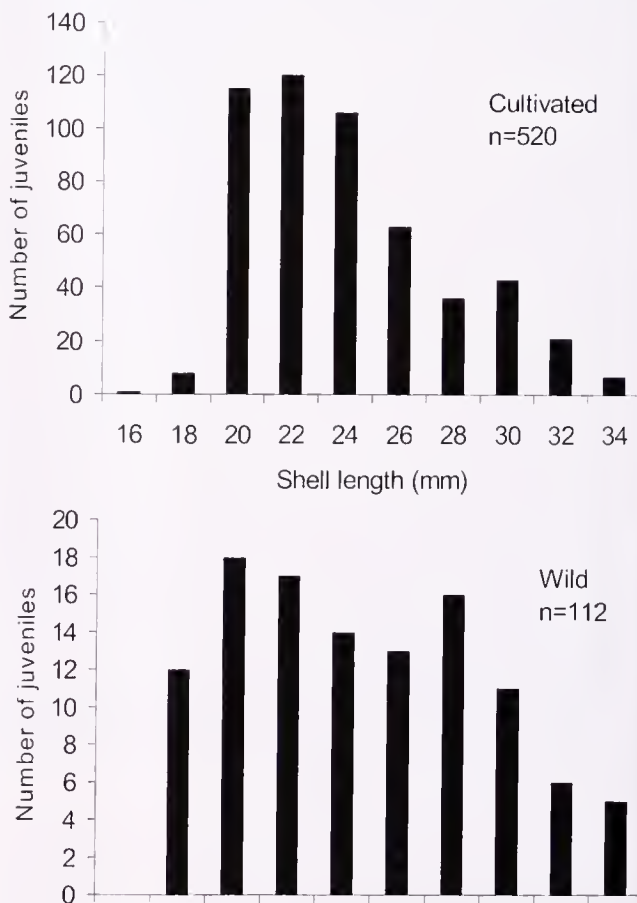
The corrected abalone counts were fitted to an exponential decay model (Rawlings 1988, Gulland 1975) to estimate the instantaneous rate of natural mortality (M) after seeding, from January 2001 to January 2003. An ANCOVA analysis was run to compare statistical significance of the wild and cultivated slopes (Scheffler 1980).

RESULTS

To be able to compare growth and survival estimates in both populations, in the case of wild abalone only specimens whose initial size range was between 17 and 34 mm SL ($n = 112$; mean length = 24.8 ± 0.87 mm) were considered in the analysis. We assumed this range included individuals approximately 1 year old, as in the case of the cultivated specimens which size ranged between 16 to 34 mm length ($n = 520$; mean length 24 ± 0.33 mm). Figure 2 indicates the size-frequency distributions of the wild and cultivated specimens used for the analysis.

Growth

Figure 3 shows the mean annual growth of wild and cultivated juveniles of *H. fulgens*. In general, both groups attained 147 ± 8 mm in length 3 years after release. Because juveniles were released when they were 1 year old, at 4 years both populations were

Figure 2. Size frequency distribution of the wild and cultivated juveniles abalone (*H. fulgens*) selected for analysis.

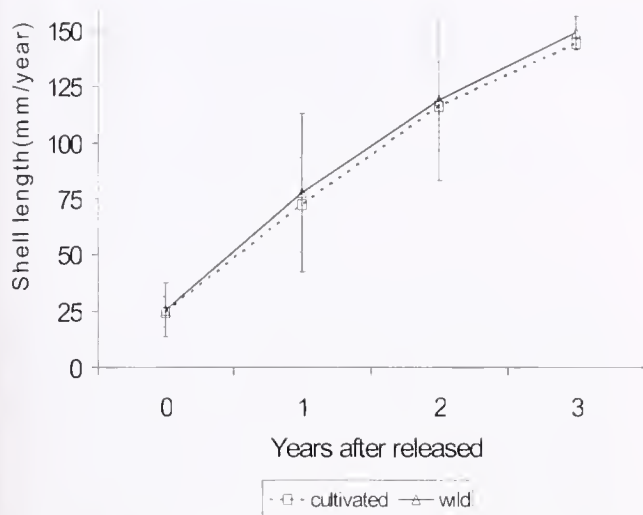


Figure 3. Mean annual growth of wild and cultivated juveniles of *H. fulgens* after seeding. Year 0 correspond to the start of the experimental period.

virtually ready to recruit to the fishery, which has a commercial minimum size of 145 mm for *H. fulgens* (Ortiz-Quintanilla & Carballo 1988).

The growth rates observed in the first year following release (at age 2 y) were 52.4 mm/year in wild juveniles and 48.1 mm/year in cultivated juveniles. These rates decreased to 29.8 and 28.1 mm/year, respectively, in the third year following release (at age 4 y) (Fig. 4). No significant difference was found between the average growth of the wild and cultured abalones ($F_{(0.05,1,3)} = 0.3633$, $P < 0.0001$).

The following equations indicate the fit of the data to the Gompertz growth model

$$Lt = 2.34 * e^{4.30(1 - e^{-0.827 * t})} \quad \text{for wild abalone}$$

$$Lt = 2.14 * e^{4.38(1 - e^{-0.812 * t})} \quad \text{for cultivated abalone}$$

Both equations look similar and the small differences observed are not significant, as ANCOVA showed in growth rates comparison (Fig. 4).

Survival

Taking into account the total number of recaptured specimens over the 3 years of the experiment, 81.6% on average were recap-

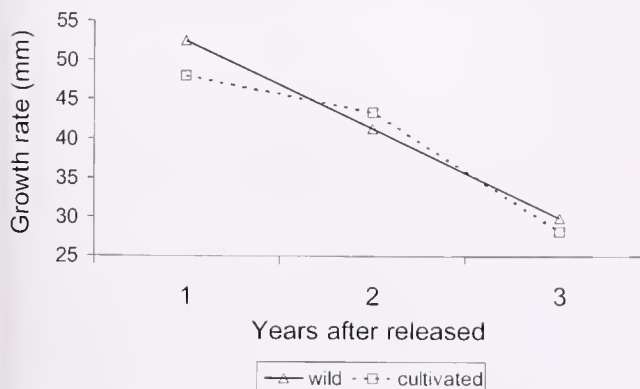


Figure 4. Annual growth rates of transplanted juveniles of *H. fulgens*.

tured once (81.8% wild and 81% cultivated), 17.2% twice (13.6% and 19%, respectively) and only 1.1% of specimens were recaptured on all three occasions (4.5% and 0%, respectively).

The number of tagged empty shells recaptured over the course of the study was very small. Tag-induced mortality or stress involved only 2 organisms 24 hours after tagging. Six months later two other empty shells were found, but these were attributed to predation.

All the recaptures of tagged specimens were found in cryptic locations. In general, specimens showed limited dispersal over the first year, the maximum magnitude of movement with respect to the release area being 2 to 4 m. Dispersal rose slightly in the second year, from 4 to 7 m, whereas by the third year, some specimens were found to have migrated up to 15 m beyond the site of their initial release. At 1.5 years after the start of the experiment, three specimens turned up in commercial catch about 50 m away from their release sites, at 3 m or 4 m depth. This suggests that as the experiment proceeded some specimens moved gradually toward deeper water. Because the search area was restricted to the lowest tide line, individuals that may have moved into the subtidal zone, like those turning up in commercial catch, would not have been recorded.

Table 1 shows the total number of recaptured abalone, including sighted and not sighted ones, after seeding in January 2000. Based on these figures the estimated values of sighting efficiency rate were: 74.2% for cultivated and 74.3% for wild juveniles.

The rates of natural mortality, calculated after seeding, from January 2001 to January 2003, were $M = 1.20 \text{ y}^{-1}$ for cultivated juveniles and $M = 0.87 \text{ y}^{-1}$ for wild juveniles (Fig. 5). ANCOVA test indicated no significant difference between the 2 mortality rates ($F_{(0.05,1,3)} = 0.0185$, $P < 0.0001$). Mortality values converted to annual survival rates were $S = 30\%$ for cultivated juveniles and $S = 42\%$ for wild juveniles.

DISCUSSION

Growth

The mean increments observed 1 year after release (48 mm in cultivated specimens and 53 mm in wild ones) indicate a high rate of growth for this species. In exceptional cases, some specimens showed increments higher than 90 mm after the first year following release.

These values exceed previous growth estimates (35 mm/year) reported for 2-year-old *H. fulgens* juveniles in the area of Bahía Tortugas (Turrubiates 1989). In experiments involving the tagging of individuals larger than 80 mm, Shepherd et al. (1991) found increments ranging from 10–40 mm/year. These growth rates are similar to those reported by Guzmán del Prío and Lopez-Salas (1993) for this same species in Bahía Asunción: 10 to 45 mm/year for abalone larger than 70 mm in length.

The rapid growth observed for both cultivated and wild specimens in the Clam Bay area may be explained by the fact that this site is an optimal habitat for this species, offering a broad availability and abundance of suitable food algae. On the other hand, the intertidal shelf where the experiment took place is of low relief and has many overlapping rocks favoring entrapment of *Macrocystis* and other drift algae which are easily transported to the hollows and crevices formed by the juxtaposition of rocks in this area (Carreón-Palau et al., 2003). Japanese workers have observed large clusters of *Haliotis* at depths where drift algae are abundant, and high densities of juveniles in shallow habitats with

TABLE 1.

Survey Date	Cultivated Juveniles				Wild Juveniles			
	Sighted	Not Sighted	Total	Sighting Proportion	Sighted	Not Sighted	Total	Sighting Proportion
Jan. 2001	48	19	67	0.72	15	8	23	0.65
Jan. 2002	25	5	30	0.83	9	2	11	0.82
Jan. 2003	4	2	6	0.67	3	1	4	0.75

pebbles (Inoue 1973, Kojima 1974, cited by Seki & Taniguchi 2000).

The observed growth rates, along with the existing data on maturity and fecundity of this species (Guzmán del Prío 1992, Gluyas-Millán & Talavera-Maya 2003), allow us to estimate that recruitment to the adult stock could take place at age 4 years, (i.e., when specimens attain a mean SL of 145 mm). Based on this, the 1-year-old juveniles currently being seeded on natural reefs may be expected to enter the fishery 3 years later. However, it should be pointed out that it would be safer for the fishery, to capture abalone equal to or larger than 160 mm SL (Shepherd et al. 1991), because although *H. fulgens* shows the first maturity signs since 105 to 115 mm (García Juárez & Ortiz Quintanilla 1992, Guzmán del Prío et al. 1980, Martínez 2003), catching *H. fulgens* bigger than 145 mm will be assuring several spawning events have taken place and with higher fecundity levels too (Martínez 2003).

Survival

The mortality values obtained in this study of 0.87 y^{-1} for wild juveniles and 1.2 y^{-1} for cultivated abalone fall well within the range from 0.2 to 4.6 for different species, reported by Shepherd (1998). Because we do not have accurate measurements of the proportion of the seeds that moved out of the area, these values might be better considered as a disappearance rate, which includes mortality and migration.

The equivalent annual survival rates for these values: 30% for

cultivated abalone seeds and 42% for wild abalone, represent the minimum survival estimates and is likely to be an underestimate because, as we said earlier, we neither have data of juveniles moved away nor enough extra data as empty shell or broken shell collections of the reef that could adjust our estimates. This scarcity of empty or broken shell collections during the surveys could be explained by our 6-month-apart surveys (shells mean half-life is 48 days, as occur in other species like *H. laevigata* [Shepherd 1998]), which made it difficult to find them.

The survival estimates obtained in these types of tag-recapture experiments tend to be biased, because they are affected by diverse factors: some mortality due to the deleterious effect of the type of tag, disturbance as a result of handling during seeding and recapture, and the fact that abalone tend to disperse when disturbed (Day & Flenting 1992, Officer et al. 2001). A further source of bias is the error as a result of low effectiveness in the search of tagged specimens (Shepherd & Breen 1992).

In our experiment, the negative effect of the tags was kept to a minimum by fixing them to the shell without affecting the respiratory pores or other structures. Nevertheless, despite the small number of empty shells recaptured (only 2), we do not rule out the possibility of some mortality being induced by seeding and subsequent recapture.

One of the major concerns was the effectiveness of searches. In our case, the sighting efficiency may have been diminished by the difficulty of turning over large-sized rocks. Despite the fact that the juveniles were seeded on movable rocks, the search had to be extended to the largest possible number of neighboring rocks, including some which were difficult to move. On the other hand, despite the care taken in moving the rocks, each search operation probably caused some dispersal of tagged specimens and an indeterminate number of them may have escaped our sight within the release area.

Another factor was the algal cover, mainly *Macrocystis* canopy, which was very thick at times and made thorough searching in certain areas difficult, particularly in summer. Hence, for the final survival estimates we have used only the annual data for each winter.

A salient fact in the experiment was the vast capacity for dispersal and crypsis shown by both wild and cultivated juveniles. It is well known that juvenile abalone are highly mobile and cryptic (Roger-Bennet & Pearce 1998). At the start of the experiment, most specimens were sighted near the release site, but as time went on it was observed that some of them (Shepherd et al. 1991) and other species moved into deeper water (Tarr 1995).

The fact that 3 adult-sized specimens (2 cultivated and 1 wild) turned up in commercial catch (1.5 years after released) indicates that some tagged specimens moved beyond the original seeding area. Using this data to get some value of the migration rate, we estimate 0.4% for cultivated and 0.8% for wild abalones. However,

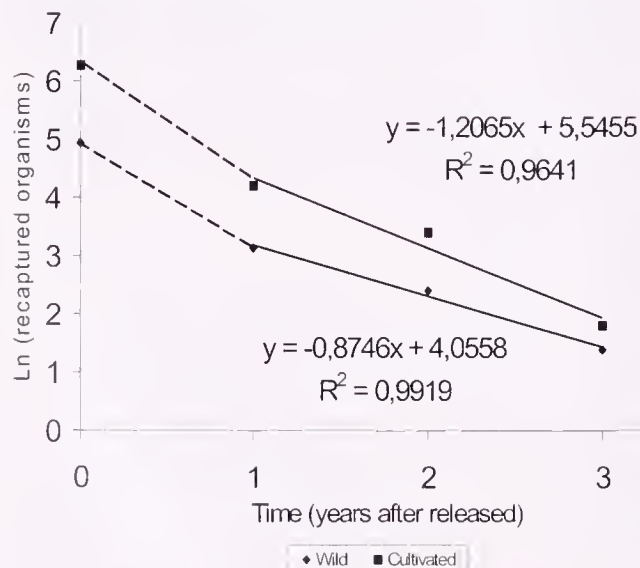


Figure 5. Mortality instantaneous rate of wild and cultivated transplanted juveniles of *Haliotis fulgens*.

these proportions hardly could be considered representative of the real migration rate values, because the specimens appeared only by chance in the commercial catch and not after an addressed survey.

Laboratory experiments have shown that wild juveniles are better able to find cryptic shelter and avoid predation than cultivated juveniles (Tegner & Butler 1989, Schiel & Weldon 1987 cited by Roger-Bennet & Pearce 1998). We did not find this difference. The sighting proportion showed almost identical values (74.2% and 74.3%) for both kind of juveniles.

We suppose that by extending the experiment period to 3 years, the probability of dispersal increased and the likelihood of sighting an indeterminate number of specimens therefore declined. Thus, the survival values reported in this study may actually be underestimates and suggest the need to perform further experiments with a different experimental design to estimate more accurately how many disperse out of the search area.

One of the major concerns of juvenile abalone seeding programs that aim to enhance the numbers on natural reefs is to increase survival and minimize production cost (Tegner & Butler 1985). Efforts to rebuild depleted or declining abalone stocks have been a concern of abalone-producing countries for several years and have furthered work on the transplanting of laboratory juveniles in Japan (Kojima 1995), the United States, (Rogers-Bennett & Pearce 1998), New Zealand (Sweijd et al. 1998), and Australia (Dixon et al. in press). In some cases, there have been transplants with relative success (Kojima 1995, Dixon et al. in press, Schiel 1993) but nevertheless, most attempts have had poor results to date (Withler 2000, Tegner & Butler 1985).

In our case, even admitting the possibility of error due to underestimation, our results do not hold out great expectations for programs aimed at recovering currently depleted stocks. We believe that at least hundreds of thousands of seeds, more than 25 mm, would have to be planted to maximize survival by seeding more robust individuals (Sweijd et al. 1998, Inoue 1976, cited by Kojima 1995). This action could ensure that a greater number of survivors impact on the production in later generations leading to

an increase in biomass; however, the production cost rise with age at release (Sweijd et al. 1998). In addition, we must consider the effects of the sudden introduction of a large number of juveniles, which may not necessarily result in increased biomass, because these specimens may be subjected to competition or predation through density-dependent predation (Hilborn 1998, cited by Shepherd et al. 2000). In other instances, they may produce an aggregative numerical response in predators (Dajoz 1996, Smith 1996), because of the number of prey, in this case the seeded juveniles, increases (Shepherd et al. 2000).

On the other hand, as long as the genetic structure of *Haliotis* populations and how it is affected by massive introductions of hatchery-reared juveniles is not well understood (Withler 2000), and whether the seed will promote recovery of stocks, it will be difficult to achieve success in attempts to enhance or recover depleted stocks. However, this issue is really beyond the scope of this study.

Therefore, at the present time, it seems that in view of the cost, and uncertainty about the benefits offered by seeding, it is wiser to manage existing populations sustainably. This involves an understanding of metapopulation structure of exploited population and requires minimum adult densities in the region. About this, Shepherd and Partington (1995) suggest for Australian waters minimum densities of 0.2–0.3 adults/m² in source habitats, but we still should determine our own figures.

ACKNOWLEDGMENTS

The authors thank the aqua culturist technicians of the Sociedad Cooperativa de Producción Pesquera Bahía Tortugas for their support with field work and the Centro Regional de Investigación Pesquera de La Paz who allowed us to use their facilities in Bahía Tortugas Laboratory. The authors also thank Rob Day and Scoresby Shepherd for their helpful critical review that improved the manuscript. This work received financial support by Instituto Politécnico Nacional through the project grants CGEPI 200494 and CGEPI 20020627.

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COMPARATIVE KARYOTYPES OF TWO NORTHEASTERN PACIFIC ABALONE SPECIES (*HALIOTIS FULGENS* PHILIPPI AND *HALIOTIS RUFESCENS* SWAINSON)

NORMIA K. HERNÁNDEZ-IBARRA,¹ CARLOS MÁRQUEZ,² JOSÉ L. RAMÍREZ¹ AND
ANA M. IBARRA^{1,*}

¹Centro de Investigaciones Biológicas del Noroeste, S.C., Lab. de Genética Acuicola A.P. 128, La Paz B.C.S., México 23000; ²Universidad Autónoma de Baja California, Facultad de Ciencias, Carr. Tijuana, Ensenada Km. 106, Ensenada B.C., Mexico 22800

ABSTRACT The karyotypes of 2 northeastern Pacific abalone species, *Haliotis fulgens* and *H. rufescens*, were evaluated from trochophore larvae cells, finding that for both species the diploid number is 36. There were differences between the two species in karyotype according to centromere position classification. *H. fulgens* karyotype was composed by 10 pairs of metacentric, 3 pairs of metacentric-submetacentric, 4 pairs of submetacentric, and 1 pair of submetacentric-subtelocentric chromosomes, and *H. rufescens* karyotypes by 8 pairs of metacentric, 6 pairs of metacentric-submetacentric, 3 pairs of submetacentric, and 1 pair of submetacentric-subtelocentric chromosomes. The relative length of chromosomes 1 to 18 was not different between the species, but 5 of their chromosomes (numbers 4, 7, 9, 11, & 18) were significantly different in relative lengths of short and long arm and in centromeric index, differences that can be explained by pericentric inversions. These observed differences in karyotype between the two species support the previous contention of evolutionary divergence by lysine studies. The chromosome number of the two species evaluated was the same than that reported for other abalones distributed in the northeast Pacific (*H. cracherodii*) and other abalone species distributed in the west Pacific (*H. discus*, *H. gigantea*, and *H. madaka*), although as between these two species, the karyotype of those previously described was also different.

KEY WORDS: chromosomes, karyotype, centromeric-index, relative-length, arm-ratio, Haliotidae

INTRODUCTION

Abalones grouped within the genus *Haliotis*, Linnaeus 1758, comprise from 60 to 70 species described worldwide (Lindberg 1992). They are distributed in the northeast Pacific, northwest Pacific, tropical west Pacific or Indo-Pacific, southwest Pacific, and Mediterranean Sea (Lindberg 1992, Lee & Vacquier 1995).

Karyotypes in abalones have been studied only for few of those 60 to 70 described species. Those species include *H. cracherodii*, from the northeast Pacific (Minkler 1977) with a diploid chromosome number $2n = 36$; *H. discus*, *H. discus hannai*, *H. gigantea*, and *H. madaka* from the northwest Pacific (Arai et al. 1982, Nakamura 1986, Wang et al. 1988, Okumura et al. 1995 in Okumura et al. 1999, Miyaki et al. 1997, Miyaki et al. 1999, Okumura et al. 1999) with a $2n = 36$; *H. varia*, *H. planata*, *H. diversicolor aquatilis*, *H. diversicolor diversicolor*, *H. ovina*, and *H. asinina* from the Indo-Pacific or tropical west Pacific (Nakamura 1985, Nakamura 1986, Arai et al. 1988, Jarayabhand et al. 1998) with a $2n = 32$; and for *H. tuberculata* from the Mediterranean Sea (Colomera & Tagliaferri 1983, Arai & Wilkins 1986) with a $2n = 28$. The knowledge of karyotypes in abalone species could provide useful information for systematics of abalone species, and also with a tool to determine the most adequate species for hybridization. Hybridization has been reported to occur among abalone species within certain geographic areas (Owen et al. 1971, Leighton & Lewis 1982, Koike et al. 1988, Hoshikawa et al. 1998), and it represents a potentially important tool for genetic improvement of abalones.

In this research, we aim to define the karyotype of two abalone species distributed in the northeast Pacific, green (or blue) abalone (*Haliotis fulgens*) and red abalone (*Haliotis rufescens*). The karyotypes for these species have not been described, but it was previously reported that both hybridize (Leighton & Lewis 1982).

MATERIALS AND METHODS

For both species, adult abalone was induced to spawn at 2 different abalone hatcheries. For red abalone, 8 cultured organisms at the commercial laboratory "BC Abalone" in Eréndira, Baja California, Mexico were used, and for green abalone 8 wild adults kept at the Laboratory of Abalone Spat Production from "Cooperativa de Buzos y Pescadores" at Isla Natividad, BCS, Mexico. They were induced to spawn, eggs fertilized, and the first hatching trochophore larvae were collected for this study. For both species, only spawns that produced normal and viable larvae were used to collect samples.

Miyaki et al. (1997) procedures for treating larvae and making slides were used. In short, colchicine (0.1%) was applied to the larvae for 2 h, applying a hypotonic shock with sodium citrate (1%) immediately after for 40 min. Larvae were fixed in Carnoy solution, exchanging this solution 3 times every 15 min. Slides were made by placing 50 to 100 larvae on a clean slide, adding 2 drops of cooled acetic acid (60%), macerating with a surgical knife to obtain a cell suspension, and dropping on this cell suspension from a height of 10–15 cm Carnoy solution to expand the macerated cells on the slide. The slides were heated and allowed to dry for approximately 1 h before staining with Giemsa (10%) for 10 min.

Chromosome counts were done at 100X using an Olympus BX41 microscope. A total of 59 metaphases and 60 metaphases were counted for red abalone and for green abalone respectively.

Karyotypes and quantitative measurements of chromosomes for both species were done using the best 10 metaphases obtained from each abalone species. Each metaphase was selected from different slides to guarantee they were derived from different larvae. Each metaphase was digitalized using a Cool SNAP-Pro (Media Cybernetics) camera, and transferred to an image analyses program (Image-Pro Plus 4.5 Program). Measurements of short and long arms of each chromosome, and mean relative length (RL) of chromosomes were estimated according to Thiriot-Quievreux

* Corresponding author: E-mail aibarra@cibnor.mx

(1984), and centromeric indices (CI) and long and short arms relative lengths (LA, SA) based on Levan et al. (1964). Classification of chromosomes was done using centromeric index according to Levan et al. (1964), using conventional names (m for metacentric, sm for submetacentric, st for subtelocentric). Those chromosomes whose mean CI fell within the limit of different types of chromosomes were classified as intermediates between the 2 classes (i.e., m-sm & sm-st). The fundamental number was estimated as White (1973), assigning the value of 4 to all metacentric, submetacentric, and subtelocentric chromosomes, and a value of 2 to telocentric pairs.

Chromosomes comparisons between the two species were done by means of a karyo-ideogram (Spotorno 1985), plotting the relative length of the short versus the long arm with their confidence intervals ($n = 10$, $\alpha = 0.05$).

RESULTS

From the 60 metaphases evaluated for *H. fulgens*, 85% had a diploid chromosome number $2n = 36$, and for the 59 metaphases in *H. rufescens* 61% a $2n = 36$ (Fig. 1).

The karyotype and a representative metaphase from which they were compared for each species are presented in Figure 2a (*H. fulgens*) & 2b (*H. rufescens*). For *H. fulgens* the relative length of the largest chromosome was 7.14 and that of the smallest chromosome was 4.45. For *H. rufescens* the relative length of the largest chromosome was 6.71 and that of the smallest chromosome was 4.48 (Table 1). The fundamental number for both species was 72.

Based on the karyo-idiogram, the karyotype of *H. fulgens* (Fig. 3) can be classified as consisting of 10 pairs of metacentric chromosomes, 3 pairs metacentric-submetacentric, 4 pairs submetacentric, and 1 pair submetacentric-subtelocentric (tending to subtelocentric because of its confidence interval). No telocentric chromosomes were observed. The karyotype of *H. rufescens* is different than that in *H. fulgens*, because it consists of 8 pairs of metacentric chromosomes, 6 pairs metacentric-submetacentric, 3 pairs submetacentric, and 1 pair submetacentric-subtelocentric. As with *H. fulgens*, no telocentric chromosomes were observed for *H. rufescens*.

The analysis by the karyo-ideogram comparing relative lengths of short and long arms, and an analysis of chromosomes differences in centromeric index between the two species indicated con-

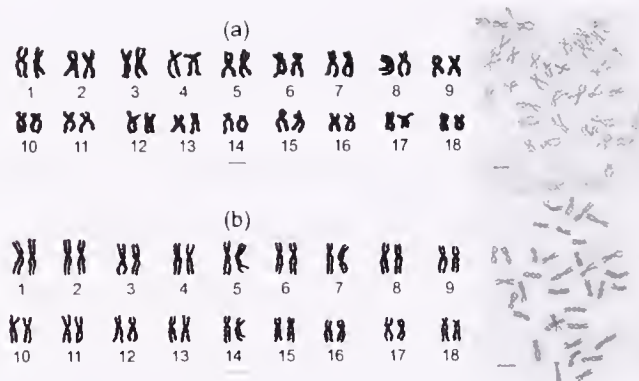


Figure 2. Karyotypes of *Haliotis fulgens* (a) and *Haliotis rufescens* (b), and metaphases used to obtain karyotypes. The bar represents 5 μ m.

sistent significant ($P < 0.05$) differences in relative lengths of short and long arms and in centromeric index between 5 of the 18 chromosomes (4, 7, 9, 11, & 18). Other chromosomes differed in CI or in one or other of the arms lengths, but not in all three (Table 1, Fig. 3).

DISCUSSION

From the abalone species for which karyotypes have been published, the smallest diploid number has been $2n = 28$, followed by an intermediate diploid number of $2n = 32$, and the largest diploid number reported being $2n = 36$ (Table 2). The diploid number of abalone species for which chromosomal studies have been done follows a pattern associated with the geographic distribution of the species (Nakamura 1986), with a $2n = 28$ seen for the Mediterranean species *H. tuberculata* (also named *H. lamellosa*), a $2n = 32$ being present among species of the Indo-Pacific or tropical west Pacific, which includes *H. varia*, *H. planata*, *H. diversicolor aquatilis*, *H. diversicolor diversicolor*, *H. asinina*, and *H. ovina*, and the largest $2n = 36$ seen for species described for the northeast and northwest Pacific, as *H. madaka*, *H. discus*, *H. gigantea*, *H. discus hannai*, and *H. cracherodii* (Table 2).

Based on genetic studies, today it is believed that not all those described abalone species are different. For example, lysine cDNA sequence homology among 27 *Haliotis* species has indicated that some of the described abalone species around the world are, in fact, the same species (Lee & Vacquier 1995). Species that are possibly a single one and are included in Table 2 are, for the Pacific northwest *H. madaka* and *H. discus hannai*; for the Indo-Pacific *H. diversicolor supertexta* and *H. diversicolor aquatilis*; and from the Mediterranean *H. tuberculata tuberculata* and *H. tuberculata lamellosa* (or *H. lamellosa*). Two additional genetic studies based on isozyme analyses have also suggested that *H. madaka* and *H. discus* are the same species (Hara & Fujio 1992 in Lee & Vacquier 1995), and that *H. diversicolor diversicolor* is different from *H. planata* and *H. varia* (Arai et al. 1988). Differences in karyotypes seem to confirm that *H. madaka*, *H. discus* and *H. discus hannai* are the same species, and that *H. diversicolor diversicolor* is different from *H. planata* and *H. varia* (Table 2).

The diploid chromosome number, $2n = 36$ of the two species evaluated in the present study, *H. rufescens* and *H. fulgens*, is in agreement with the previously reported karyotype for only one species from the northeast Pacific, *H. cracherodii* (Minkler 1977), and the reported association between the diploid number with geographic distribution (Nakamura 1986). In as much as the northwest Pacific (Japan) abalone species also have a diploid number of 36,

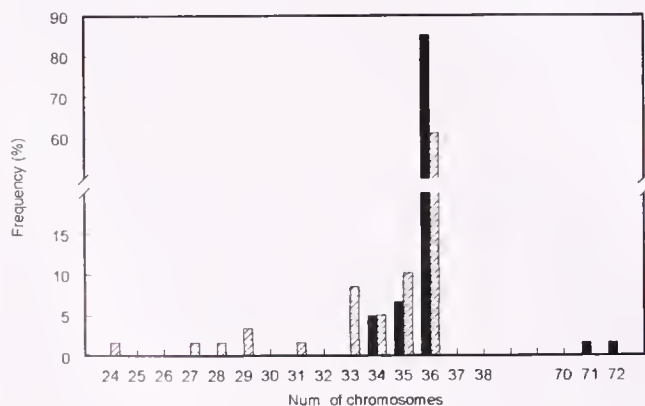


Figure 1. Frequency distribution of total number of chromosomes observed in metaphases of cells from trochophore larvae for *Haliotis fulgens* ($n = 59$) dark bars, and *Haliotis rufescens* ($n = 60$) light bars.

TABLE 1.

Karyotype comparison of *Haliotis fulgens* (H.f.) and *Haliotis rufescens* (H.r.): relative length RL, short arm length SA, long arm length LA, centromeric index CI, and chromosome classification derived from 10 metaphases for each species. Standard deviations are presented (\pm sd) for each mean.

Chrom pair	RL \pm sd		SA \pm sd		LA \pm sd		IC \pm sd		C	
	H.f.	H.r.	H.f.	H.r.	H.f.	H.r.	H.f.	H.r.	H.f.	H.r.
1	7.14 \pm 0.38 ^a	6.71 \pm 0.51 ^a	2.97 \pm 0.13 ^a	3.18 \pm 0.22 ^a	4.17 \pm 0.32 ^a	3.53 \pm 0.33 ^a	41.70 \pm 1.85 ^a	47.40 \pm 1.44 ^b	m	m
2	6.77 \pm 0.59 ^a	6.68 \pm 0.31 ^a	2.53 \pm 0.29 ^a	2.88 \pm 0.17 ^a	4.24 \pm 0.38 ^a	3.80 \pm 0.19 ^a	37.32 \pm 2.44 ^a	43.10 \pm 1.36 ^b	m-sm	m
3	6.70 \pm 0.34 ^a	6.63 \pm 0.40 ^a	3.11 \pm 0.19 ^a	3.02 \pm 0.23 ^a	3.59 \pm 0.19 ^a	3.61 \pm 0.19 ^a	46.38 \pm 1.41 ^a	45.55 \pm 1.29 ^a	m	m
*4	6.55 \pm 0.33 ^a	6.23 \pm 0.66 ^a	1.96 \pm 0.16 ^a	2.44 \pm 0.20 ^b	4.59 \pm 0.32 ^a	3.79 \pm 0.52 ^b	29.92 \pm 2.29 ^a	39.33 \pm 2.72 ^b	sm	m-sm
5	6.43 \pm 0.53 ^a	6.09 \pm 0.45 ^a	2.88 \pm 0.25 ^a	2.07 \pm 0.16 ^b	3.55 \pm 0.31 ^a	4.02 \pm 0.39 ^a	44.83 \pm 1.39 ^a	34.10 \pm 2.43 ^b	m	sm
6	6.05 \pm 0.28 ^a	6.08 \pm 0.45 ^a	1.91 \pm 0.18 ^a	2.26 \pm 0.23 ^a	4.13 \pm 0.16 ^a	3.83 \pm 0.27 ^a	31.59 \pm 1.78 ^a	37.07 \pm 1.71 ^b	sm	sm-m
*7	5.79 \pm 0.46 ^a	5.93 \pm 0.22 ^a	2.22 \pm 0.17 ^a	1.81 \pm 0.18 ^b	3.57 \pm 0.32 ^a	4.11 \pm 0.20 ^b	38.40 \pm 1.41 ^a	30.54 \pm 2.64 ^b	m-sm	sm
8	5.70 \pm 0.55 ^a	5.88 \pm 0.45 ^a	2.06 \pm 0.24 ^a	2.52 \pm 0.21 ^b	3.64 \pm 0.36 ^a	3.36 \pm 0.38 ^a	36.07 \pm 2.05 ^a	42.97 \pm 3.17 ^b	sm	m
*9	5.55 \pm 0.26 ^a	5.64 \pm 0.25 ^a	2.64 \pm 0.17 ^a	2.18 \pm 0.18 ^b	2.91 \pm 0.14 ^a	3.46 \pm 0.11 ^b	47.63 \pm 1.52 ^a	38.66 \pm 1.63 ^b	m	m-sm
10	5.27 \pm 0.26 ^a	5.42 \pm 0.24 ^a	1.73 \pm 0.10 ^a	2.26 \pm 0.11 ^b	3.55 \pm 0.19 ^a	3.16 \pm 0.22 ^a	32.76 \pm 1.14 ^a	41.80 \pm 2.13 ^b	sm	m
*11	5.22 \pm 0.35 ^a	5.15 \pm 0.33 ^a	2.11 \pm 0.14 ^a	2.47 \pm 0.13 ^b	3.12 \pm 0.25 ^a	2.68 \pm 0.21 ^b	40.37 \pm 1.75 ^a	48.03 \pm 0.87 ^b	m	m
12	5.19 \pm 0.28 ^a	5.13 \pm 0.24 ^a	2.27 \pm 0.15 ^a	1.98 \pm 0.09 ^b	2.92 \pm 0.16 ^a	3.15 \pm 0.17 ^a	43.79 \pm 1.33 ^a	38.62 \pm 1.05 ^b	m	m-sm
13	4.80 \pm 0.19 ^a	5.09 \pm 0.25 ^a	1.96 \pm 0.13 ^a	2.24 \pm 0.12 ^b	2.84 \pm 0.14 ^a	2.85 \pm 0.19 ^a	40.80 \pm 1.95 ^a	43.96 \pm 1.91 ^a	m	m
14	4.68 \pm 0.25 ^a	5.06 \pm 0.18 ^a	1.26 \pm 0.13 ^a	1.74 \pm 0.05 ^b	3.42 \pm 0.18 ^a	3.32 \pm 0.17 ^a	26.86 \pm 1.95 ^a	34.45 \pm 1.26 ^b	sm-st	sm
15	4.64 \pm 0.28 ^a	4.61 \pm 0.20 ^a	1.69 \pm 0.10 ^a	1.66 \pm 0.11 ^a	2.95 \pm 0.22 ^a	2.95 \pm 0.13 ^a	36.45 \pm 1.64 ^a	36.07 \pm 1.42 ^b	sm-m	sm-m
16	4.55 \pm 0.35 ^a	4.60 \pm 0.22 ^a	2.12 \pm 0.21 ^a	1.90 \pm 0.10 ^a	2.43 \pm 0.15 ^a	2.70 \pm 0.16 ^a	46.58 \pm 1.46 ^a	41.37 \pm 1.42 ^b	m	m
17	4.53 \pm 0.25 ^a	4.59 \pm 0.26 ^a	2.18 \pm 0.12 ^a	2.11 \pm 0.13 ^a	2.35 \pm 0.16 ^a	2.48 \pm 0.15 ^a	48.07 \pm 1.34 ^a	46.0 \pm 1.38 ^a	m	m
*18	4.45 \pm 0.27 ^a	4.48 \pm 0.15 ^a	1.85 \pm 0.13 ^a	1.13 \pm 0.07 ^b	2.59 \pm 0.18 ^a	3.35 \pm 0.14 ^b	41.67 \pm 1.59 ^a	25.14 \pm 1.32 ^b	m	sm-st

* indicates that the chromosomes between the two species are different in SA, LA, and IC. Different letters between species indicate significant differences based on confidence intervals ($P < 0.05$). m, metacentric; sm, submetacentric; st, subtelocentric.

our results confirm that marked differences in karyotypes do exist between northwest and northeast Pacific abalones. Northwest Pacific abalones only have metacentric and submetacentric chromosomes, whereas northeast Pacific abalones have those types and additionally have intermediates between those types, and two (Minkler 1977) telocentric pairs of chromosomes, or one submetacentric-subtelocentric in this study. The importance of defining chromosome types as intermediates between two classes comes

from different conclusions reached in different studies for the same species. For example, northwest abalone species described for northwest Pacific are very homogeneous in number of metacentric and submetacentric chromosomes, with most reports indicating that there are 10 pairs of metacentric and 8 pairs of submetacentric chromosomes in *H. discus* (*H. discus discus*, *H. discus hannai*, and *H. madaka*), and *H. gigantea* (Arai et al. 1982, Okumura et al. 1995 in Okumura et al. 1999, Miyaki et al. 1997, Miyaki et al. 1999). However, there are also two reports (Wang et al. 1988, Okumura et al. 1999) indicating 11 pairs of metacentric and 7 pairs of submetacentric for *H. discus* (*H. discus hannai*). The differences reported for *H. discus* (*H. discus hannai*) have been attributed to three pairs of chromosomes (4, 9, and 17) that have arm ratios close to the limits of the classification between metacentric and submetacentric (Okumura et al. 1999), resulting in ambiguous classification even within which today is believed to be the same species.

Contrasting the homogeneity in types of chromosomes for northwest Pacific species, the northeast Pacific species evaluated so far indicate less homology in the numbers of metacentric (m), submetacentric (sm), and subtelocentric (st) chromosomes between species (*H. cracherodii* with 8 m: 8 sm: 2 st, but *H. fulgens* with 10 m: 3 m-sm: 4 sm: 1 sm-st, and *H. rufescens* with 8 m: 6 m-sm: 3 sm: 1 sm-st. Lack of homology in karyotypes has also been reported for the Indo-Pacific species studied so far, which are characterized by a diploid chromosome number of $2n = 32$. However, lack of homology in karyotypes has been reported also for what is now believed (Lee & Vacquier 1995) to be the same species (Table 2). This result can be explained by the reduced sample size used for karyotyping, or by using low quality metaphases. For example, within this group of Indo-Pacific abalone species, two conflicting karyotypes have been reported within

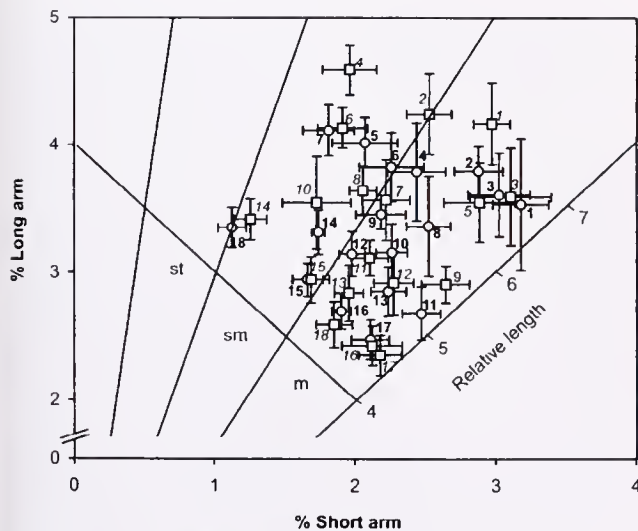


Figure 3. Karyo-idiogram of *H. fulgens* and *H. rufescens*. Relative lengths and confidence intervals of each species chromosome short and long arms are plotted: *Haliotis fulgens*, squares; *Haliotis rufescens*, circles. Chromosome classification, m = metacentric, sm = submetacentric, st = subtelocentric.

TABLE 2.
World abalone species evaluated for their karyotype.

Distribution	Species	s	n	2n	FN	m	m-sm	sm	sm-st	st	t	RL ¹	CI ¹	AR ¹	RL ^s	CI ^s	AR ^s	Reference
European-Mediterranean ¹	<i>H. tuberculata</i>			28	56	8		6										Arai & Wilkins 1986
	"		14	28														Colombero & Tagliaferri 1983
	<i>H. Lamellosa</i>		14	28*														
Indo-Pacific ²	<i>H. varia</i>		16	32	64*	13		3										Nakamura 1986
	"			32	64*	9		6	1									Arai et al. 1988
	"			32	64*	8		8										Jarayabhand et al. 1998
	<i>H. planata</i>			32	64*	9		6	1									Arai et al. 1988
	<i>H. diversicolor</i>		16	32	64*	8		5	2	1								Nakamura 1985
	<i>H. diversicolor aquatilis</i>																	
	<i>H. diversicolor diversicolor</i>			32	64*	8		7		1								Arai et al. 1988
	<i>H. asinuma</i>			32	64*	10		6										Jarayabhand et al. 1998
Pacific Northwest ³	<i>H. ovina</i>			32	62*	9		6			1							Jarayabhand et al. 1998
	<i>H. discus</i>			36	72*	10		8										Arai et al. 1982
	"			36	72	10		8										Miyaki et al. 1997
	"			36	72	10		8										Miyaki et al. 1997
	<i>H. discus hamai</i>			36	72*	10		8										Arai et al. 1982
	"			36	72	11		7				7.09			4.11			Wang et al. 1988
	"			36	72*	10		8										Okumura et al. 1995
	"			36	72*	11		7				6.88		1.21	4.64		1.10	in Okumura et al. 1999
	<i>H. madaka</i>			36	72	10		8										Miyaki et al. 1999
	<i>H. gigantea</i>		18	36*														Nakamura 1986
Pacific Northeast ³				36	72	10		8										Miyaki et al. 1997
	<i>H. cracherodii</i>			36	72*	8		8		2								Minkler 1977
	<i>H. fulgens</i>			36	72	10	3	4	1			7.14	41.70	1.40	4.45	41.67	1.41	Present study
	<i>H. rufescens</i>			36	72	8	6	3	1			6.71	47.40	1.11	4.48	25.14	3.01	Present study

(1) Eurotis, (2) Padollus, and (3) Nordotis: subgenera subdivision within the Haliotidae (Lee & Vacquier 1995).

FN fundamental number, m metacentric, sm submetacentric, st subtelocentric, t telocentric, RL relative length, CI centromeric index, AR arm ratio, ¹ largest chromosome, ^s smallest chromosome.

* data inferred (for FN: m, sm, st = 4, and for t = 2).

(S) indicates within the same block species which have been later found to be the same one.

two species, *H. varia* (Nakamura 1986, Arai et al. 1988, Jarayabhand et al. 1998) and *H. diversicolor* (Nakamura 1985, Arai et al. 1988). Nakamura (1986) states that his data for *H. varia* are not precise because of the small size (1.41 to 3.63 μ m) of some of the chromosomes he found, and Jarayabhand et al. (1998) attributes the difference in his study with that in Arai et al. (1988) to different methodological conditions. However, the microphotographs of *H. varia* karyotype included in Jarayabhand et al. (1998) indicate a possible problem with measurements because of the small chromosome size and a low definition of metaphases. The differences reported for karyotypes within *H. diversicolor* can be explained by one author classifying 2 chromosomes as submetacentric, whereas the other one classifies those chromosomes as submetacentric-subtelocentric, indicating that the centromeric index is in the limits of both classes, pointing again to the need for more detailed classification when the chromosome centromeric indices are in, or close to the limits of two classifications.

Based on the results obtained in this study, indicating that whereas the chromosomes of *H. rufescens* and *H. fulgens* do not differ in their relative length (RL), that 5 of the 18 chromosomes were different in centromeric index (CI), relative length of short arm, and relative length of long arm, it is possible to propose causes for the differences in chromosome structure between these two species. For all of those chromosomes that were different (4,

7, 9, 11, and 18), pericentric inversions can be proposed to be the evolutionary force differentiating the two species. Pericentric inversions represent a structural chromosome change known to be the most frequent mutations in the evolution of karyotypes (White 1978). Finally, another important difference between the karyotypes of the two species is the position of the only submetacentric-subtelocentric chromosome in each, based on decreasing RLs, which is in position on chromosome number 14 for *H. fulgens* and position on chromosome number 18 for *H. rufescens*, suggests a possible translocation event in the common lineage of these species.

A characteristic among abalone species reported in the literature is the presumable existence of hybrids. The occurrence of hybridization has been inferred from morphologic characteristics in natural populations (Owen et al. 1971) or from mating experiments in the laboratory (Leighton & Lewis 1982, Koike et al. 1988, Hoshikawa et al. 1998). However, genetic confirmation of hybridization has only been done for hybrids of *H. kamtschatica* and *H. discus hamai* (Hoshikawa et al. 1998). Given the karyotype differences observed in this study it is possible to conclude that even if fertilization between these two species can be achieved producing hybrids, those hybrids would be expected to have a reduced fertility because of improper synapses of not perfectly homologous chromosomes during meiosis (Freeman & Herron 2001). In agreement, Leighton & Lewis (1982) found a low vi-

ability (0.1%) to postlarva when mating (presumed, not genetically certified) hybrids of red and green abalone.

ACKNOWLEDGMENTS

The authors thank the abalone laboratories BC Abalone, Erendira and Cooperativa de Buzos y Pescadores Isla, Nativi-

dad for the donation of biological material, and also Susana Avila from CIBNOR for laboratory technical support. The authors also thank Dr. Nick Elliot (CSIRO) and anonymous reviewers for valuable comments on the manuscript. This research was supported by CONACYT grant 38860-B to A. M. Ibarra.

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A COMPARATIVE EVALUATION OF THE HABITAT VALUE OF SHELLFISH AQUACULTURE GEAR, SUBMERGED AQUATIC VEGETATION AND A NON-VEGETATED SEABED

JOSEPH T. DEALTERIS, BRIAN D. KILPATRICK, AND ROBERT B. RHEAULT

Department of Fisheries and Aquaculture, University of Rhode Island, Kingston, RI 02881

ABSTRACT The habitat value of modified rack and bag, shellfish aquaculture gear (SAG) used for the grow-out phase of the American oyster, *Crassostrea virginica*, submerged aquatic vegetation (SAV), *Zostera marina*, and a shallow nonvegetated seabed (NVSB) was comparatively evaluated over a 1-year period in Pt. Judith Pond, a tidal estuary in Southern Rhode Island. Enclosure gear was used to sample the three ecotypes, and organisms (>5 mm) were identified, enumerated, and measured to the nearest millimeter. Abundances of marine organisms and species diversity indices were used as measures of the habitat value of these ecotypes within each season. Environmental and geological parameters were not significantly different between the habitats. Emergent surface area ($\text{cm}^2 \text{ m}^{-2}$ of seabed) within each ecotype was estimated, and used to evaluate its role in providing habitat. The SAG habitat had a significantly greater surface area than either the SAV or NVSB habitats during all seasons. The physical structure of the SAG habitat protects juvenile fish from predators and provides substrate for sessile invertebrates that serve as forage for fish and invertebrates. The SAG habitat supported a significantly higher abundance of organisms per m^2 of seabed throughout the year. Species richness was also significantly greater in the SAG habitat compared with the SAV and NVSB habitats. A 2-way ANOVA indicated significant differences in species diversity (Shannon-Weiner index) between habitats. Tukey's HSD test indicated that the SAG habitat had significantly higher species diversity than the NVSB habitat, but no significant difference in species diversity was found between the SAG and SAV habitats. These findings indicate that shellfish aquaculture gear provides habitat for many organisms throughout the year, and is especially beneficial to ecosystems that support native species of recreationally and commercially important fish and invertebrates in their early life history stages. Therefore, we conclude that shellfish aquaculture gear has substantially greater habitat value than a shallow nonvegetated seabed, and has habitat value at least equal to and possibly superior to submerged aquatic vegetation.

KEY WORDS: shellfish aquaculture, habitat value, submerged aquatic vegetation

INTRODUCTION

Habitat is the place where an organism lives (Odum 1971). This simple definition is the basis for most ecologic studies involving habitat quality or value. Other considerations regarding the definition of habitat are that an organism at any particular life stage has only one habitat and that an organism's spatial distribution defines its habitat (Minello 1999). The characteristics of habitat that have been identified as being beneficial to organisms include physical structure, provision of food, substrate, hydrodynamics, and hydrology, and these must be specified to quantify habitat utilization by a particular species (Minello 1999). Physical structure is provided by submerged aquatic vegetation (SAV) or man-made structures like artificial reefs. The terms habitat "value" or "quality" when pertaining to fishery resources is defined as a habitat's ability to support a fishery resource (finfish, crustaceans, molluscs, and all other forms of marine animal and plant life). Studies that describe fishery resource habitat value primarily use species density or abundance data (Able, 1999). The purpose of this study is to comparatively evaluate the habitat value of modified rack and bag, shellfish aquaculture gear (SAG) used for the grow-out phase (Rheault & Rice 1995) of the American oyster, *Crassostrea virginica*, submerged aquatic vegetation (SAV), *Zostera marina*, and a shallow nonvegetated seabed (NVSB) over a 1-year period in Pt. Judith Pond, a tidal estuary in southern Rhode Island. The SAG habitat uniquely supplies an abundance of substrate due to the wire racks and rigid, plastic bags, in addition to the shell of the cultivated oyster.

In a study designed to estimate relative habitat value, Smith et al. (1989) used mark-recapture data and estimated densities of scallops (*Argopecten irradians*) to compare a recently transplanted eelgrass (*Zostera marina*) bed to a natural eelgrass bed. In a similar study, Fonseca et al. (1996) used abundances of shrimp, fish, and crab species to assess habitat value of the replanted eelgrass as compared with nonvegetated areas and naturally occurring eel-

grass meadows. Recent studies involving oyster reefs have used similar criteria to determine relative habitat value by sampling nekton densities within the reefs. Coen et al. (1999a) conducted a long-term study comparing the habitat value of oyster reefs in the southeastern United States by measuring several parameters, including water quality and abundances, of resident and transient fauna. Faunal densities were used to compare species richness between natural and experimental reefs. Carr and Hixon (1997) compared fish assemblages and abundances to determine species richness on natural and artificial reefs. O'Beirn et al. (2001) investigated the organisms associated with oysters cultured in floating systems by measuring the number of macro-faunal species inhabiting these floating culture systems, so as to determine the species richness of this unique habitat.

Natural oyster reefs have been identified as essential fish habitat because not only do they support the oysters themselves but a myriad of other fishery resources. There is abundant evidence that indicates these reef communities are extremely diverse and show differences in species abundances as compared with adjacent non-vegetated, sand flat habitats. Oyster reef habitats are not only highly diverse but include species absent in adjacent soft-bottom environments (Coen et al. 1999b). In addition to obligate oyster reef residents, a variety of transient species occupy the reef in a facultative way (Posey et al. 1999). Grass shrimp, blue crabs, and other fish were observed utilizing the reefs possibly for foraging or refuge purposes. Breitburg and Miller (1998) reported that resident finfish populations are dependent on oyster reef habitats due to the physical extent of the reefs, their suitability as refuges from predators, and abundance of prey for consumption. These characteristics influence the abundance, growth, and reproduction of these resident finfish, thus demonstrating that oyster reefs enhance fish production. There is evidence that the 3-dimensional structure of oyster reefs affect the spatial distribution of various fish and perhaps the overall abundances. Striped bass and other predatory fish have

been observed to hover near reefs utilizing them as foraging sites (Breithurg 1999).

Habitats that exhibit structural complexity have been shown to support higher numbers of species as compared with barren non-vegetated bottom types (Orth & Heck 1980). Orth et al. (1984) concluded that an increase in habitat complexity due to eelgrass density should increase refuges for prey species. Man-made structures have also been shown to increase abundances of fishery resources (Carr & Hixon 1997). Man-made structures or "artificial reefs" may be specially constructed and consist of concrete rubble (Kelch et al. 1999) used for the purposes of creating habitat for fish. Grossman et al. (1997) hypothesized that if habitat is limiting, new artificial reefs can potentially increase fish production through 3 mechanisms: (1) an increase of foraging habitat for adult, juvenile, and/or newly recruited fishes; (2) an increase in breeding habitat; and (3) an increase in predator refuge or resting habitat. Therefore, shellfish aquaculture gear may serve as an artificial reef habitat by virtue of its inherent structural complexity and extensive time spent on the seafloor throughout the year, thereby increasing the fish production in the ecosystem.

MATERIALS AND METHODS

Study Area

Three habitats (SAG, SAV, and NVSB) were sampled in Pt. Judith Pond, Rhode Island, a shallow 6 km tidal estuary that discharges into Block Island Sound. The 1.0 h aquaculture lease site contained over 600 oyster cages, each consisting of a 1.8 m \times 0.6 m \times 0.6 m wire cage that held 12 mesh bags of shellfish on shelves. The oyster cages were placed 2.4–6.1 m apart on the seabed in 2.4–3.0 m of water. The SAV and NVSB habitats were located approximately 1.5 km south of the aquaculture lease in Pt. Judith Pond at similar depths of water.

Experimental Design

The research design was a four (season) by three (habitat type) factorial design with three replicates within each habitat. Three habitats (SAG, SAV, and NVSB) were seasonally sampled in replicate between December 2000 and October 2001 so as to evaluate the following habitat characteristics: macro-epibenthic fauna community structure, and the physical, chemical, and geological environmental conditions. All three habitats sampled using enclosure type gears to maximize the efficiency and consistency of sampling (Rozas & Minello 1997).

Field and Laboratory Methods

Moonstone Oyster Company cultivates the American Oyster (*Crassostrea virginica*) in cages that are cleaned every 4–6 months. We selected cages for sampling that had been cleaned 4–6 weeks prior to each seasonal sampling so that they would have a representative seasonal fouling population. Lift-nets (2.1 m \times 0.9 m with a 2-mm mesh) were placed beneath three randomly selected SAG units 2 weeks before sampling to allow sufficient time for swimming organisms to return to the cages following the disturbance of lifting the cage to place the lift nets underneath.

A scuba diver deployed the lift-net so that it completely enclosed the oyster cage during recovery. All free swimming epifauna >5 mm were recovered from the lift net enclosure along with three randomly selected oyster bags, and were taken back to the laboratory for analysis. Each oyster cage was also randomly

sampled in five locations with a 0.022 m² (15 cm \times 15 cm) quadrat to assess sessile invertebrate growth. The oyster cages are constructed of 5.1 cm mesh, vinyl-coated, 2 mm diameter wire. Percent cover of each biofouling organism within each quadrat sample was assessed to the nearest class and/or phylum. The percent cover of sessile invertebrate growth on the oyster bags was determined in a similar fashion. Total biomass of sessile invertebrates on the cages and bags was estimated for the entire surface area of the cages and bags by extrapolating mean sample values to the total surface area. A random subsample of 10 oysters was taken from each of three bags taken from each cage. Oyster length and width was measured to the nearest millimeter using vernier calipers and the surface area of the oysters and sessile invertebrate growth on both sides was estimated to the nearest square centimeter. Results were averaged within seasons and extrapolated over an average of 200 oysters per bag or 2,400 oysters per cage. The total surface area and sessile invertebrate coverage (cm²) for each oyster cage consisted of the sum of the surface area of the oyster cage, the 12 oyster bags, and the seasonal average surface area of the 2,400 oysters. These sums were divided by the area enclosed by the lift net used to sample the SAG habitat (1.95 m²). Thus, surface area and invertebrate growth are referenced to area (m²) of the seabed.

The SAV and NVSB habitats were sampled on the same day within a few hours of noontime during each of the seasons. These habitats were randomly sampled using a 2-mm mesh drop-net (2.13 m \times 0.92 m) and a venturi-driven suction dredge deployed from a small skiff. The animals were collected in a 2 mm-mesh catch bag and returned to the laboratory for analysis. The emergent portion of the SAV habitat was randomly subsampled with a 0.25 m² quadrat (3 replicates) each season. The eelgrass blades within each quadrat were clipped at the base and measured to the nearest 100 cm using vernier calipers. Sessile invertebrate growth (cm²) on the SAV was similarly estimated. The NVSB habitat was devoid of emergent substrate and attached sessile invertebrates.

All free swimming organisms >5 mm in length collected from each of the three habitats were identified to the genus and species, and measured to the nearest millimeter using vernier calipers. Temperature, salinity, and dissolved oxygen were seasonally measured during each sampling event in each habitat. Sediment from each habitat was collected seasonally using a 7.5-cm diameter \times 15.2-cm deep corer. Mean sediment grain size was determined by dry sieve analysis (Folk 1968).

Data Analysis

Seasonal environmental parameters (temperature, salinity, and dissolved oxygen) were analyzed by 2-way analysis of variance (ANOVA) without replication (EXCEL 1997) between habitat and season. The environmental dependent variables for each season were also analyzed using 1-way analysis of variance (ANOVA). Tukey's honest significant difference (HSD) test was used to compare treatment means when an F-test indicated significant treatment effects (SPSS vs. 10 1999). Sediment type data for each habitat was characterized according to percent gravel, sand, and silt-clay using a 2-way ANOVA without replication (EXCEL 1997) between habitat and season. This analysis was repeated after subtracting the gravel component from the oyster cage habitat to compensate for the presence of shell hash from the aquaculture operations. Physical habitat complexity was measured in terms of emergent surface area within each habitat. The average surface area within each of the replicates for each habitat was log transformed

($\ln(\text{cm}^2)$) to satisfy the homogeneity of variance assumption for an analysis of variance (Zar 1984). The average surface area was compared with a 2-way ANOVA (SPSS vs.10 1999) between habitats and seasons, and Tukey's HSD test (SPSS vs.10 1999) was used to compare treatment means when an F-test indicated significant treatment effects.

The community structure was analyzed using Ecological Methodology (Krebs 1989) statistical software (Exeter Software 2000). The raw data used in the statistical software consisted of species abundances (3 replicates) within each habitat for each season. Species richness was determined by the Jackknife method for quadrat counts (Helshe & Forrester 1983). Shannon-Weiner species diversity and Smith and Wilson species evenness indices were generated using Ecological Methodology statistical software (Exeter Software 2000). The indices of species richness, diversity, and evenness within each habitat were each analyzed using a 2-way ANOVA (SPSS vs.10 1999) between habitat and season. Tukey's HSD test (SPSS vs.10 1999) was used to compare treatment means when an F-test indicated significant treatment effects.

Species abundance data within each habitat were compiled into 5 categories for analysis: total abundances of all organisms sampled, fish, crustacean, mollusk abundances, and total surface covered by sessile invertebrates. The abundance data were log transformed ($\ln(X)$) to satisfy the homogeneity of variances assumption (Zar 1984) and analyzed using a 2-way ANOVAs (SPSS vs.10 1999) between habitat and season for each abundance category. Tukey's HSD test (SPSS vs.10 1999) was used to compare treatment means when an F-test indicated significant treatment effects. Correlation analysis (EXCEL 1997) was used to investigate the relationship between the total abundance of animals observed in each habitat and season, and the emergent surface area found in each habitat and season.

RESULTS

Environmental Parameters and Sediment Characteristics

There were no significant differences in temperature, dissolved oxygen, or salinity between sites ($P < 0.05$) in any given season. Temperature varied seasonally from 3.0 to 23.7 °C; salinity was influenced by rainfall and ranged from 25.0 ppt to 34.6 ppt; and dissolved oxygen peaked in winter/spring at 11.9 mg/L and was lowest in spring/summer at 6.4 mg/L. The three sampling sites had a similar grain size composition, dominated by sand (mean 93.5%) and silt-clay (mean 6.5%), however there was a substantial gravel component (4.27%) in the SAG site that was comprised primarily of oyster shell fragments. After removing this fraction, the sediments from the three sites were not significantly different from each other ($P < 0.05$).

Habitat Structure

Habitat structure, described in terms of emergent surface area (cm^2) per m^2 of seabed, varied as a function of habitat type and season (Fig. 1). The log transformed average emergent surface area varied significantly both between sites and between seasons ($P < 0.001$). There were significant differences ($P < 0.05$) between each of the 3 habitats (SAG>SAV>NVSB), and significant differences ($P < 0.01$) between each of the seasons (except between spring/summer and winter/spring). The SAG habitat, due to the cages, bags, and oysters, provided an average of more than 60 times the emergent surface area per square meter over the course

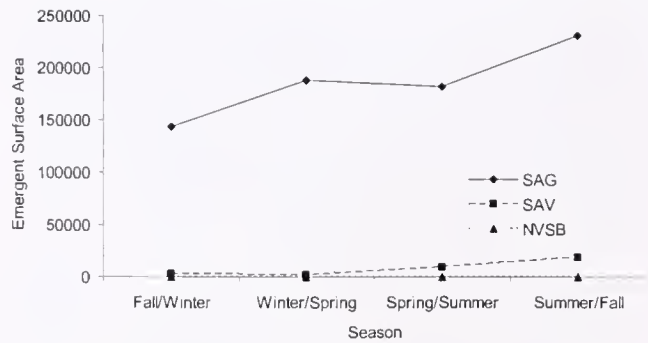


Figure 1. Emergent surface area (cm^2/m^2 of seabed) for each habitat and season.

of the year than the SAV habitat. The SAV habitat had mean shoot densities of 554/ m^2 in the spring/summer and summer/fall seasons and 224/ m^2 in the fall/winter and winter/spring seasons. The NVSB habitat was devoid of emergent surface area during all seasons.

Community Structure

Species richness was also consistently higher in the SAG habitat (Fig. 2a). There were significant differences ($P < 0.01$) between habitats, and between seasons ($P < 0.05$). Species richness was significantly different between each habitat (SAG>SAV>NVSB) and between fall/winter and summer/fall seasons. The mean Shannon-Weiner Index values of species diversity were highly significantly different between habitats ($P < 0.001$) and between

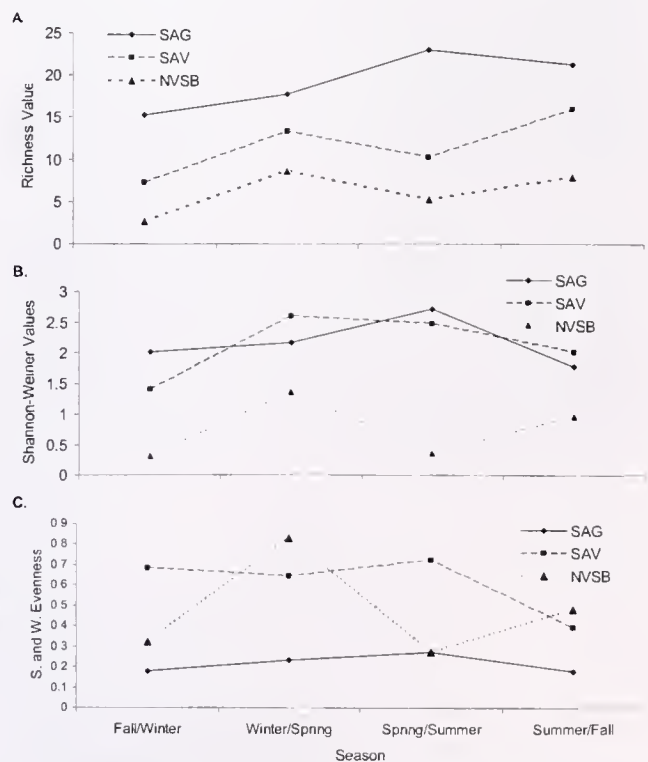


Figure 2. A. Species richness values for each habitat and season. B. Mean Shannon-Weiner values for each habitat and season. C. Mean Smith and Wilson measure of evenness values for each habitat and season.

seasons ($P < 0.01$). The SAG habitat was not significantly different from the SAV habitat ($P > 0.05$), however both of these habitats were highly significantly different ($P < 0.01$) from the NVSB (see Fig. 2b). Significant differences ($P < 0.05$) in species diversity were also found between the fall/winter and winter/spring sampling and between fall/winter and spring/summer. The SAG habitat showed consistently lower Smith and Wilson species evenness values than either the SAV or NVSB because a few species tended to dominate this habitat (see Fig. 2c). There were highly significant differences in species evenness between habitats ($P < 0.001$), but not between seasons ($P > 0.05$). The SAG habitat was significantly lower in species evenness than either the SAV or NVSB habitats ($P < 0.05$).

Species Abundances

The SAG habitat consistently supported far greater abundances of organisms than either the SAV or the NVSB habitats throughout the year (Fig. 3). There were highly significant differences ($P < 0.001$) between habitat and seasons for the species abundance data. There was a highly significant difference ($P < 0.001$) in species abundance between each habitat (SAG>SAV>NVSB). There was also a significant difference ($P < 0.05$) in species abundances between all seasons except winter/spring and spring/summer sampling periods showed no significant differences ($P > 0.05$). A strong correlation ($r = 0.94$) was found between the total abundance of organisms in each habitat and season and the emergent surface area available in corresponding habitat and season (Fig. 4).

Ten fish species were identified inhabiting one or more of the three habitats sampled during the course of the study (Fig. 5), and individual fish species abundances are shown for each habitat and season in Figure 6. There were highly significant differences ($P < 0.001$) in fish abundances between habitats and seasons. The greatest fish abundances ($P < 0.01$) occurred in the SAG habitat followed by the SAV habitat and then the NVSB habitat. The summer/fall sampling period had significantly higher ($P < 0.01$) fish abundances compared with any other season. With two exceptions, the SAG habitat supported higher abundances of fish than either SAV or NVSB habitats. The Northern Pipefish (*Syngnathus fuscus*) in the spring/summer and summer/fall and the Winter Flounder (*Pleuronectes americanus*) in the summer/fall were unique to the SAV. There were many species of fish that were unique to SAG including the American eel (*Anguilla rostrata*), oyster toadfish (*Opsanus tau*), rock gunnel (*Pholis gunnellus*), and Atlantic tomcod (*Microgadus tomcod*). Several fish species were sampled throughout each season in the SAG, which included the seaboard

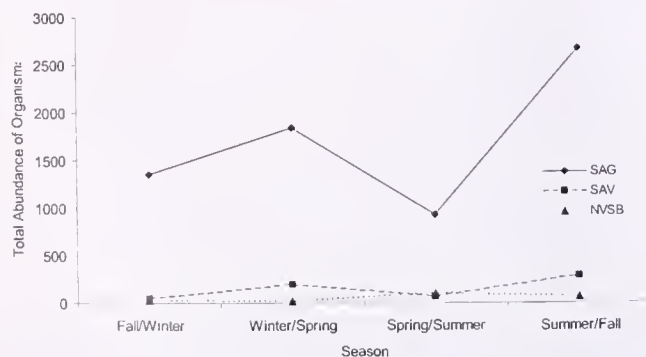


Figure 3. Total abundances of organisms collected within each habitat and season.

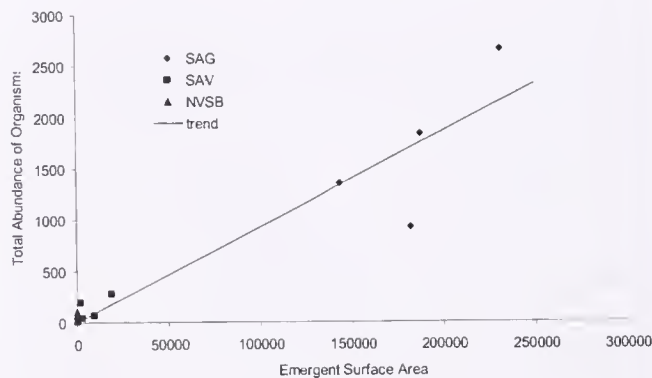


Figure 4. Correlation of total abundance of organisms (abundance) and emergent surface area (surface area cm²/m² of seabed).

goby (*Gobiosoma ginsburgi*), grubby (*Myoxocephalus aeneus*), tautog (*Tautoga onitis*), and cunner (*Tautoglabrus adspersus*). The SAG habitat was the only habitat sampled that supported one or more fish species year-round.

Thirteen crustacean species were identified to inhabit one or more of the three habitats sampled during the course of the study (Fig. 7), and individual crustacean species abundances are shown for each habitat and season in Figure 8. There were highly significant differences ($P < 0.01$) in crustacean abundances between habitats and seasons. The greatest abundances occurred in the SAG habitat followed by the SAV habitat and then the NVSB habitat. The summer/fall sampling period had significantly higher ($P < 0.01$) crustacean abundances compared with any other season. The American Lobster, *Homarus americanus*, was the only crustacean unique to the SAG habitat (5 observed individuals). The average carapace length was 6.3 cm (S.E. \pm 0.88), which places these lobsters in the juvenile phase of their lifecycle (Hudon 1987).

Seven mollusk species were identified to inhabit one or more of the three habitats sampled during the course of the study (Fig. 9), and individual mollusk species abundances are shown for each habitat and season in Figure 10. There were highly significant differences ($P < 0.01$) in mollusk abundances between habitats and seasons. The greatest abundances occurred in the SAG habitat followed by the SAV habitat and then the NVSB habitats. The winter/spring sampling period had significantly higher ($P < 0.01$) crustacean abundances compared with any other season.

Sessile invertebrate species were present in both SAG and SAV habitats (Fig. 11). The NVSB habitat was devoid of surface and hence the absence of sessile invertebrates. Statistics were not performed to detect differences between habitats due to the high variability of sessile invertebrate abundances.

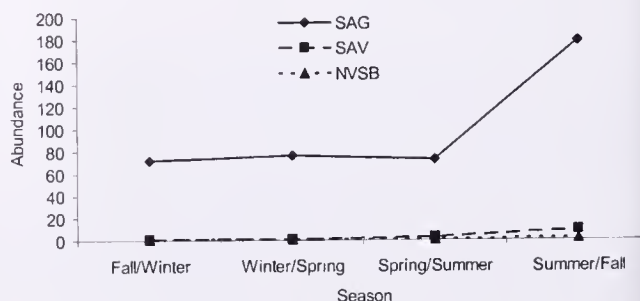


Figure 5. Total fish abundances found within each habitat and season.

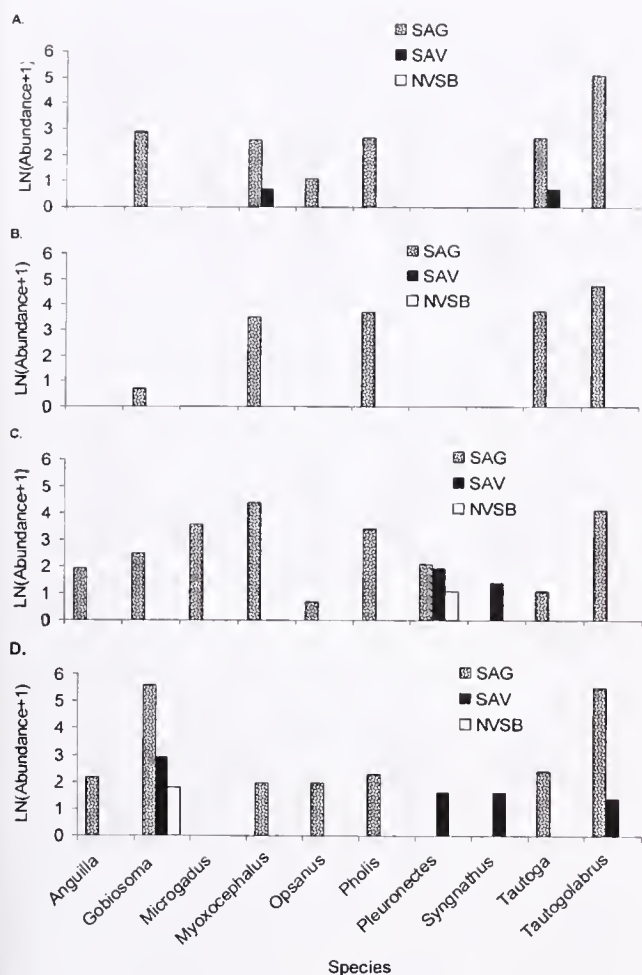


Figure 6. Total abundances (ln(abundance+1)) of individual fish species found within each habitat during: A. fall/winter, B. winter/spring, C. spring-summer, and D. summer-fall sampling periods for the following species: *Anguilla rostrata*, *Gobiosoma* spp., *Microgadus tomcod*, *Myoxocephalus aeneus*, *Opsanus tau*, *Pholis gunnellus*, *Pleuronectes americanus*, *Syngnathus fuscus*, *Tautoga onitis*, *Tautoglabrus adspersus*.

DISCUSSION

Habitat is the place where an organism lives during any part of its lifecycle (Odum 1971). The ecologic value of habitat is inferred by quantifying the resident and transient marine organisms associated with a particular habitat. Consequently, the greater the abun-

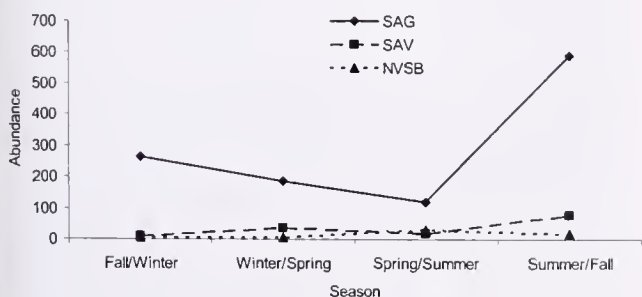


Figure 7. Total crustacean abundances found within each habitat and season.

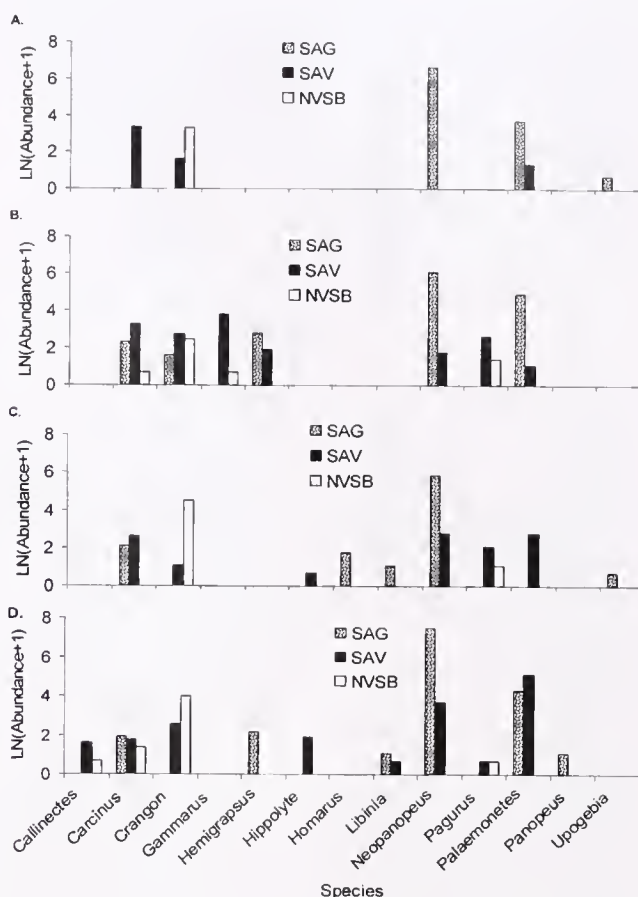


Figure 8. Total abundances (ln(abundance+1)) of individual crustacean species found within each habitat during: A. fall/winter, B. winter/spring, C. spring-summer, and D. summer-fall sampling periods for the following species: *Callinectes sapidus*, *Carcinus maenas*, *Crangon septemspinosa*, *Gammarus* spp., *Hemigrapsus sanguineus*, *Hippolyte* spp., *Homarus americanus*, *Libinia emarginata*, *Dyspanopeus sayi*, *Pagurus longicarpus*, *Panopeus* spp., *Upogebia affinis*.

dance and diversity of fish in a particular habitat, the greater its habitat value (Able 1999). SAV and natural oyster reefs have been identified as important fish habitats not only because of shelter they provide to resident and transient marine organisms, but also because of the ecologic services they provide to the surrounding environment. The objective of our study is to comparatively evaluate the habitat value of SAG, SAV and NVSB in a small estuary. The SAV habitat sampled in this study is typical of other SAV

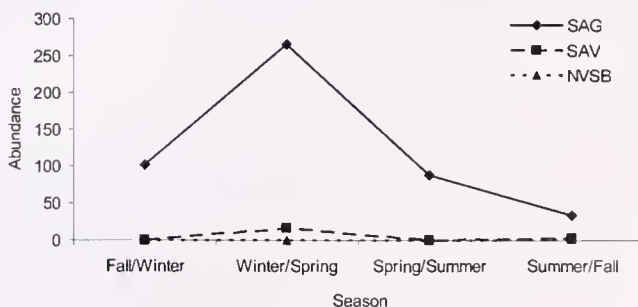


Figure 9. Total mollusk abundances found within each habitat and season.

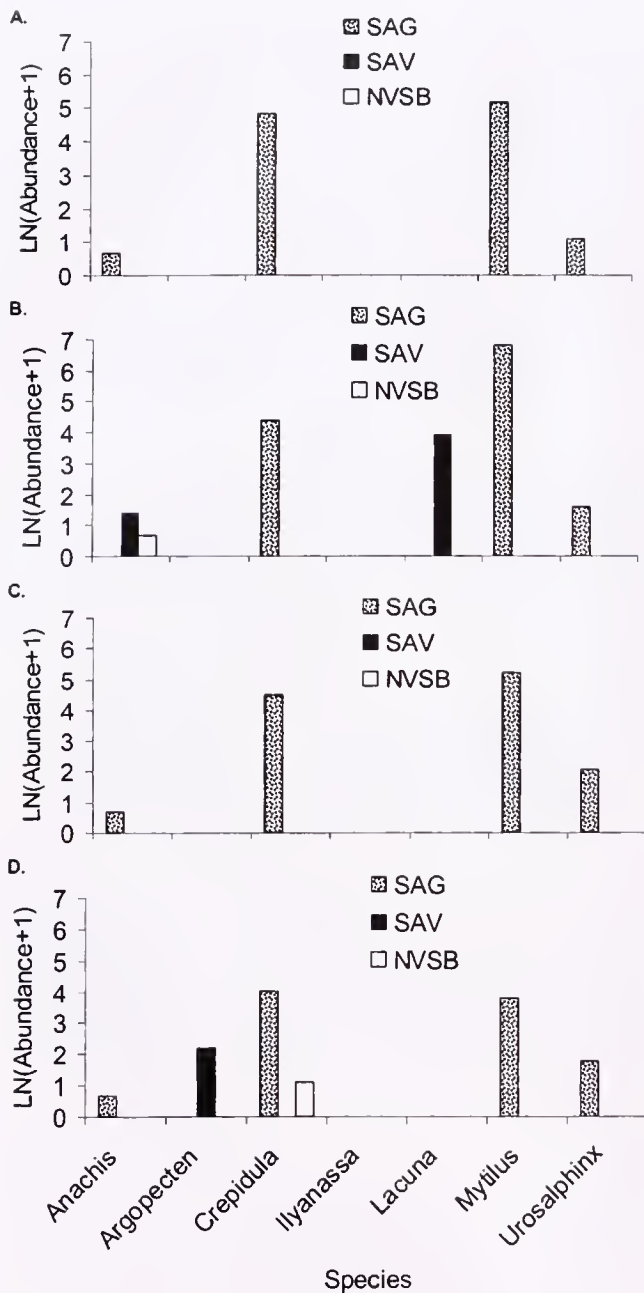


Figure 10. Total abundances (ln(abundance+1)) of individual mollusk species found within each habitat during: A. fall/winter, B. winter/spring, C. spring-summer, and D. summer-fall sampling periods for the following species: *Anachis* spp., *Argopecten irradians*, *Crepidula fornicata*, *Ilyanassa trivittata*, *Lacuna vineta*, *Mytilus edulis*, *Urosalpinx cinerea*.

habitats in New England and the mid-Atlantic regions based on eelgrass shoot density (Thayer et al. 1984).

The environmental parameters were relatively consistent among habitats within each season. No significant differences were observed between habitats for temperature, salinity, and dissolved oxygen, as was expected considering each habitat is contained within the same estuary. Also, as expected, the major differences among environmental parameters occurred between seasons. Sediment type between habitats was found to be similar after

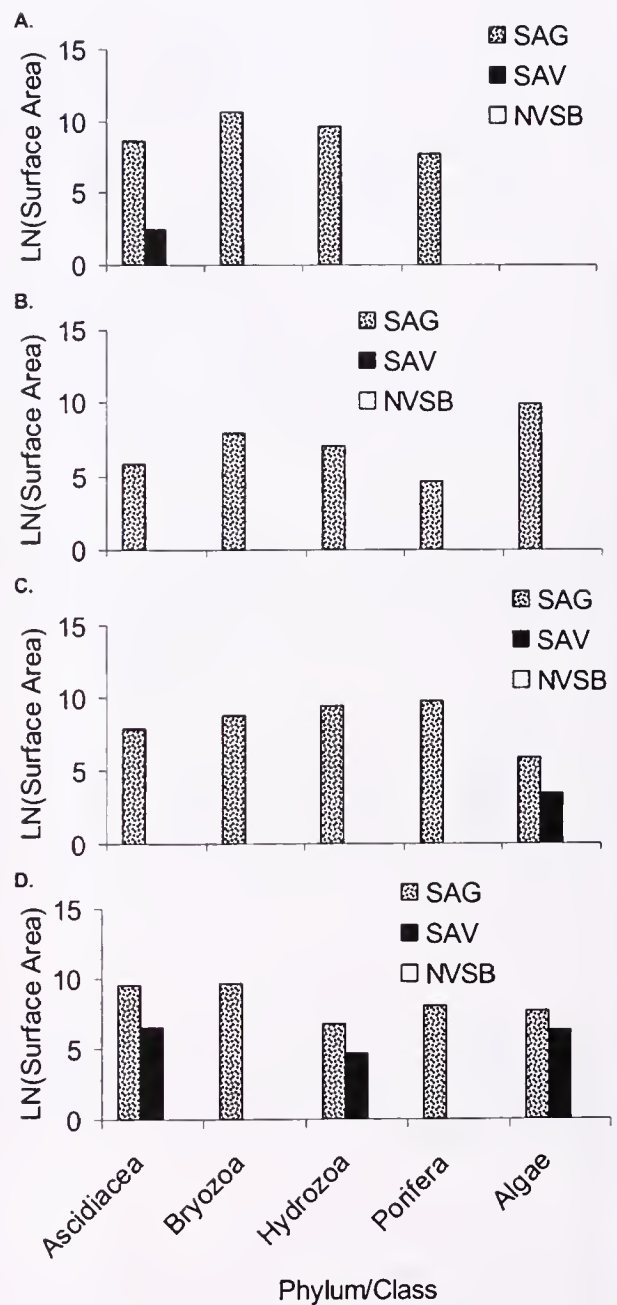


Figure 11. Total surface area (ln(surface area (cm²))) of sessile species found within each habitat during: A. fall/winter, B. winter/spring, C. spring-summer, and D. summer-fall sampling periods for the following phylum/class groups: *Ascidiacea*, *Bryozoa*, *Hydrozoa*, *Porifera*, *Algae*.

the gravel component was removed from the SAG site. The gravel component in the SAG site consisted of shell hash, which is a direct result of the aquaculture activities that take place over the seabed. The differences observed in species abundances and diversity between habitats are not likely to be related to environmental or geological parameters. Therefore, we believe that the observed differences in species composition and abundances are influenced by differences in habitat composition, structure, and complexity.

There was a highly significant difference in emergent surface

area (cm^2) between each habitat that was strongly correlated with abundance of organisms observed. The NVSB habitat supported significantly fewer organisms than either SAV or the SAG habitats throughout the year. The SAV emergent surface varied throughout the year due to seasonal growth and mortality patterns. The SAG emergent surface area varied seasonally as a result of the measured changes in the surface area of the oysters, whereas the surface area of the cages and bags remained constant. We believe that the higher abundances of species found in the SAG habitat throughout the year are related to the high surface area, the large numbers of spaces inside the cages that serve as refuge, and the prevalence of fouling organisms and forage. Structural heterogeneity was not considered when quantifying each habitat. The SAG habitat is constructed of 2-inch (5.08 cm) plastic-coated wire mesh. It can be assumed that the size of the wire mesh restricted many of the predator species of certain sizes and hence the cages became a refuge for many of the juvenile species of fish. These results are consistent with many studies that have recognized increased habitat complexity supports higher abundances of organisms due to increased predator protection (Orth et al. 1984, Ryer 1988, Beck 2000).

The high surface area within the shellfish aquaculture gear provides habitat not only for mobile fauna but also sessile biofouling invertebrates. Sponges, hydroids, bryozoans, and ascidians were found in both the SAG and SAV but the SAG habitat clearly displayed larger abundances of sessile invertebrate species. The SAV does support epiphytic and sessile invertebrate growth but not to the extent of the SAG. Although not intensively studied in this research, sessile invertebrate communities form the base of the food web for many artificial reef communities (Blancher et al. 1994). The high prevalence of sessile invertebrate communities on the SAG not only increases habitat complexity, but also increases food resources for the marine organisms inhabiting the aquaculture gear.

The SAG habitat shares many attributes and similarities with natural oyster reefs and artificial reefs. The oysters within the aquaculture gear are providing many of the same ecologic services as those found within naturally occurring oyster reefs. These ecologic services include but are not limited to particle clearance, nutrient removal and remineralization, benthic-pelagic coupling, and the creation of refuge from predators (Coen et al. 1999a, Dame 1999). The SAG also provides 3-dimensional structural complexity and many of the same benefits that artificial reefs provide in areas where habitat is limiting. Studies have shown and suggested that biologic services of artificial reefs include foraging habitat and predator refugia to resident and transient marine organisms (Blancher et al. 1994, Bohnsack 1989).

The abundance (organisms $>5\text{mm}$) and species richness exhibited in the aquaculture gear was greater than the eelgrass habitat, which in turn was greater than the unvegetated site, consistent with previous studies (Orth & Heck 1980, Mattila et al. 1999, Heck et al. 1995). This research clearly indicates more organisms inhabit the SAG habitat either SAV or NVSB habitats per square meter of seabed throughout the year. Species diversity levels were similarly higher in the shellfish aquaculture gear and the eelgrass ecotypes than in the unvegetated bottom consistent with findings of Marshall-Adams (1976), Mattila et al. (1999), Heise & Bortone (1999). Average species diversity in the SAG habitat was higher, but not significantly, than in the SAV habitat. The evenness measures

varied greatly for each habitat throughout the year, however the SAG habitat had consistently lower evenness than the other ecotypes because of the hyperdominance of several species within the aquaculture gear (*Dyspanopeus sayi*, *Tautogalabrus adspersus*, and *Mytilus edulis*). In contrast, the SAV habitat was rarely dominated by a few species, but rather supported a more equal distribution of organisms. The NVSB habitat showed a greater fluctuation of evenness values directly affected by the abundances of the sand shrimp (*Crangon septemspinosa*) sampled during each season. The sand shrimp was by far the most dominant species in the NVSB habitat and accounted for 87% of the NVSB organisms sampled throughout the year.

The abundance and species diversity data elucidate the similarities and differences between each of the three habitats. The oyster cages supported much greater species abundances than eelgrass, but displayed similar species diversity (as shown by the Shannon-Weiner index). Eelgrass is a habitat known to provide many valuable ecosystem services and has been demonstrated to be a critical and essential habitat to many commercial and recreationally important species. The species abundance and diversity data from this study suggest that the shellfish aquaculture gear has similar habitat value for its inhabitants when compared with eelgrass. The species evenness data clearly shows that whereas the abundances may be greater in the SAG habitat, the SAG habitat is dominated by a few species.

The SAG habitat may also act as a predator refuge during early life stages of the lobster due to the limiting habitat within Point Judith Pond. In the spring and summer small lobsters are regularly found in the oyster cages and large predatory fish have been observed to frequent the aquaculture lease area including: the American shad (*Alosa sapidissima*), striped bass (*Morone saxatilis*), and winter flounder (*Pleuronectes americanus*). The American lobster supports an important fishery in the northeast United States therefore any habitat found to support the lobster should be considered commercially beneficial.

There is little research to date that describes the ecosystem services and benefits of aquaculture gear and its associated cultured product. The ecosystem services of the cultured bivalves and the benefits they provide to the marine ecosystem are fundamentally similar to those provided by wild stocks of bivalves. The aquaculture gear used to grow the cultured bivalves has intrinsic habitat complexity and shares many of the characteristics that artificial reefs possess. However, aquaculture gear is not a fixed structure, but it is periodically disturbed during maintenance and harvest operations. Most SAG habitat organisms are undoubtedly displaced during cleaning operations. Some of the sessile organisms are killed, but, the mobile species are probably able to quickly relocate to another of the 600 cages nearby when they are disturbed. The maintenance and cleaning of the aquaculture gear initiates recolonization of sessile invertebrate growth and inhabitation by motile organisms.

These findings indicate that shellfish aquaculture gear provides habitat for many native species of recreationally and commercially important fish and invertebrates in their early life history stages throughout the year. Therefore, we conclude that shellfish aquaculture gear has habitat value at least equal to and possibly superior to submerged aquatic vegetation. Future research should focus on growth, survival, and production of fish biomass within this habitat to further elucidate its habitat value.

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MOLLUSCAN COMMUNITIES OF THE ROCKY INTERTIDAL ZONE AT TWO SITES WITH DIFFERENT WAVE ACTION ON ISLA LA ROQUETA, ACAPULCO, GUERRERO, MÉXICO

ARCADIO VALDÉS-GONZÁLEZ,^{1,*} PEDRO FLORES-RODRÍGUEZ,^{1,2}
RAFAEL FLORES-GARZA^{1,2} AND SERGIO GARCÍA-IBÁÑEZ¹

¹Doctorado en Ciencias Biológicas, Especialidad en Ecología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México. C. P. 66450;

²Universidad Autónoma de Guerrero, Unidad Académica de Ecología Marina, Acapulco, Guerrero, México. C. P. 39390

ABSTRACT Molluscan communities were studied on two shores, one exposed and the other sheltered from wave action by determining the diversity, richness, density, dominance, and similarity of the groups of species found in the upper part of middle intertidal zone. The sample area per location and date was 20 m². The samples were taken quarterly during the year. Species richness was found to be 44 species for both sites combined. The exposed site had a greater richness (41 species) and lower density (64.2 organisms/m²), whereas the sheltered site showed a higher density (88.08 organisms/m²) and smaller richness (33 species). Thirty species (68.8%) were determined to be common. The exposed shore had more confined species. The Shannon-Wiener diversity index (*H'*) was higher for the exposed site (3.165 bits/individual). A comparison of means by the Mann-Whitney tested indicated that the annual means of *H'* were equal between the two sites. Qualitative (Jaccard) and quantitative (Morisita-Horn) similarity scores were high at 0.682 and 0.622, respectively. The mean density of gastropods was the same at both locations, because bivalves showed significant differences with higher densities at the sheltered site. The malacologic community of the two sites was very similar. However, changes in richness and diversity over time were greater at the sheltered shore, while these changes were more gradual at the exposed shore, reflecting a greater stability of the malacologic community at the latter location.

KEY WORDS: molluscan communities, rocky intertidal, wave action, Acapulco

INTRODUCTION

The Pacific rocky coast has a very variable relief, which determines microenvironmental conditions at each location, which in turn control the distribution of species. Molluscs stand out because of their abundance and variety of forms, and have been connected with the development of many cultures.

It is important to measure some of the ecological attributes of the communities, so that comparison can be made with species found at other locations. The information also has practical application for the purpose of management, appropriate use, conservation, and environmental monitoring. On the overall geographic scale, climate is the principal factor that determines the type of communities, whereas on a smaller scale it is more difficult to establish which factors govern the grouping of species. Various physical factors are known to intervene in the shaping of the structure of communities, one of them being exposure to waves. Studies of molluscan populations and communities along the Mexican Pacific have historically focused on species of commercial interest (Baquero & Stuardo 1977, Baquero 1979, Ríos-Jara et al. 2001), and the majority of these have been conducted on the continental shelf (Reguero & García-Cubas 1989, Landa-Jaime & Arciniegas-Flores 1998). There have been few investigations in the particular case of rocky shores, where most of the authors have carried out taxonomic works (Morris 1966, Keen 1971, Holguín-Quinones & González-Pedraza 1989, 1994, Sevilla 1995, Reyes-Gómez & Salcedo-Vargas 2002), and others have been concerned with the zonation, richness and species diversity of molluscs (Villalpando-Canchola 1986, Salcedo-Martínez et al. 1988, Castillo-Rodríguez & Amezcua-Linares 1992, Román-Contreras et al. 1991, Esqueda et al. 2000, Villarreal et al. 2000) and the different regions exposed

to waves (García-López 1994, Del Río-Zaragoza & Villarreal-Melo 2001).

The main economic activity in the State of Guerrero is tourism and to a lesser degree fishing. However, there is little known about marine faunistic resources, despite that it involves an extensive coastline that encompasses three marine regions prioritized for the conservation of the coastal and ocean biodiversity of Mexico, and Mexico's National Commission on Biodiversity (CONABIO) has indicated that there is a lack of knowledge about this region (Arriaga et al. 1998). Isla La Roqueta is located within this zone.

The aim of this investigation is to determine the diversity, density, dominance, and similarity among the groups of mollusc species in the high intertidal zone of a shore exposed to wave action and one not, under the supposition that locations that differ in exposure also differ in composition of the malacologic community.

METHODS

Area of Study

Isla La Roqueta is located in Acapulco, Guerrero, and the sample sites were as follows: (a) Playa Zoológico is situated 16°42'11.2"N and 99°54'8.8"W, and is a shore exposed to wave action. Its massif is composed of intrusive igneous rocks, and has a firm substrate with a large amount of fissures, cracks, and cavities all over the area; (b) Playa Palmitas is situated 16°49'25.6"N and 99°54'41.2"W. It is a sheltered shore with a massif composed of intrusive igneous rocks. It has a firm substrate with a large amount of fissures, cracks, and cavities. The climate is hot and sub-humid, with rains in the summer and with 5% to 10% winter rain (García 1981).

Field Methods

The sample was taken from the upper part of middle intertidal zone of the two locations. The sampling dates were December of

*E-mail: acuacu_uanl@yahoo.com.mx

2000 and March, June, September and December of 2001. The calculated area for the sample size was 20 m² for each station, determined by the method of species area (Brower et al. 1998). Collection was at 2 vertical levels of the upper part of the middle intertidal zone, called level I with its lower limit adjacent to the barnacle zone, and level II with its upper limit next to the periwinkle and limpet zone. A strip 30 m long and 2 m wide was used, which was parallel to the coastline, and the sampling unit was a quadrant 1 m squared. Sampling was systematic (Scheaffer et al. 1987), randomly selecting the starting point and placing the quadrant in level I. After finishing the first sampling unit, the quadrant was placed in level II, directly above level I. All the molluscs that were found in the quadrant were identified and counted. After completing both levels, a space of 2 m was left before establishing new quadrants until completing 20 m².

Taxonomic identification of the molluscs was according to Morris (1966), Keen (1971), Skoglund (1991, 1992), Reyes-Gómez and Salcedo-Vargas (2002).

Analysis of Data

The density of the molluscs was determined as number of organisms/m². Species richness constituted the number of species present per site. A species was considered dominant if it showed a density equal to or greater than 1 organism/m² in at least one of the samplings. Cumulative dominance was defined as the sum of individual dominances. The frequency of appearance was deter-

mined as the total number of samples in which each species was recorded and expressed as a percentage. A confined species was defined as the only species present in one of the sites. The difference between means of species richness was evaluated with the Mann-Whitney test (Daniel 2002). Similarity between the sites was measured by using the qualitative presence/absence index of Jaccard and the quantitative Morisita-Horn index based on abundance (Magurran 1989). Species diversity was determined with the Shannon-Wiener index H' (Margalef 1974), and the Mann-Whitney test was used to compare the mean values of diversity H' (Daniel, 2002).

RESULTS

Exposed Shore

A total of 41 species were found (Table 1) corresponding to 31 genera and 19 families, of which 75.6% belonged to the class Gastropoda, 17.1% to the class Bivalvia, and 7.3% to the class Polyplacophora. The lowest richness was found in the samplings of March and June with 21 species and the greatest in December of 2001 with 26 species (Fig. 1).

The mean density during the annual cycle was 64.2 organisms/m², which fluctuated among the different sampling times from 29.9 to 87.3 organisms/m².

Sixteen dominant species were recorded and in the annual cycle these species accumulated to 98.02% dominance. The quantity of

TABLE 1.

Molluscan species observed at both locations of collecting sites in the upper part of middle intertidal zone on Isla la Roqueta, Acapulco, Guerrero, México; Dec. 2000–Dec. 2001.

Gastropoda	
1. <i>Cantharus sanguinolentus</i> (Duclos, 1833) ^C	18. <i>Mitra tristis</i> Broderip, 1836 ^C
2. <i>Columbella fuscata</i> Sowerby, 1832 ^C	19. <i>Mitrella ocellata</i> (Gmelin, 1791) ^C
3. <i>Conus nux</i> Broderip, 1833 ^S	20. <i>Nerita scabricosta</i> Lamarck, 1822 ^C
4. <i>Crucibulum scutellatum</i> (Wood, 1928) ^C	21. <i>Nodilittorina aspera</i> (Philippi 1846) ^C
5. <i>Crepidula excavata</i> (Broderip, 1834) ^E	22. <i>Nodilittorina modesta</i> (Philippi, 1846) ^C
6. <i>Diodora inaequalis</i> (Sowerby, 1835) ^E	23. <i>Opeatostoma pseudodon</i> (Burrow, 1815) ^C
7. <i>Fissurella asperella</i> Sowerby, 1835 ^E	24. <i>Petalocochus complicatus</i> Dall, 1908 ^C
8. <i>Fissurella gemmata</i> Menke, 1847 ^C	25. <i>Petalocochus macrophragma</i> Carpenter, 1857 ^C
9. <i>Fissurella nigrocincta</i> Carpenter, 1856 ^C	26. <i>Planaxis obsoletus</i> Menke, 1851 ^S
10. <i>Fissurella obscura</i> Sowerby, 1835 ^E	27. <i>Plicopurpura columellaris</i> (Lamarck, 1822) ^C
11. <i>Hoffmannella hansii</i> Marcus & Marcus, 1967 ^C	28. <i>Plicopurpura patula pansa</i> (Gould, 1853) ^C
12. <i>Leucozonia cerata</i> (Wood, 1828) ^C	29. <i>Siphonaria gigas</i> Sowerby, 1825 [†]
13. <i>Lottia acutapex</i> (Berry, 1960) ^E	30. <i>Siphonaria palmata</i> Carpenter, 1857 ^C
14. <i>Lottia mitella</i> (Menke, 1847) ^E	31. <i>Stramonita haemastoma</i> (Linnaeus, 1758) ^C
15. <i>Lottia pediculus</i> (Philippi, 1846) [†]	32. <i>Tectura fascicularis</i> (Menke, 1851) ^C
16. <i>Mancinella speciosa</i> (Valenciennes, 1832) ^C	33. <i>Thais kioskiiformis</i> (Duclos, 1832) ^C
17. <i>Mancinella triangularis</i> (Blainville, 1832) ^C	34. <i>Trimusculus stellatus</i> (Sowerby, 1835) ^S
Bivalvia	
35. <i>Arca mutabilis</i> (Sowerby, 1833) ^E	39. <i>Isognomom janus</i> Carpenter, 1857 ^C
36. <i>Brachidontes semilaevis</i> (Menke, 1849) ^C	40. <i>Striostrea prismatica</i> (Gray, 1825) ^C
37. <i>Chama equinata</i> Broderip, 1835 ^C	41. <i>Saccostrea palmula</i> (Carpenter, 1857) ^C
38. <i>Choromytilus palliopus</i> (Carpenter, 1857) ^C	
Polyplacophora	
42. <i>Cliton albolineatus</i> Broderip y Sowerby, 1829 [†]	44. <i>Tonicia forbesi</i> Carpenter 1857 ^E
43. <i>Cliton articulatus</i> , Sowerby, 1832 ^C	

^CCommon species in both sites. [†]Confined species in exposed site. ^SConfined species in sheltered site.

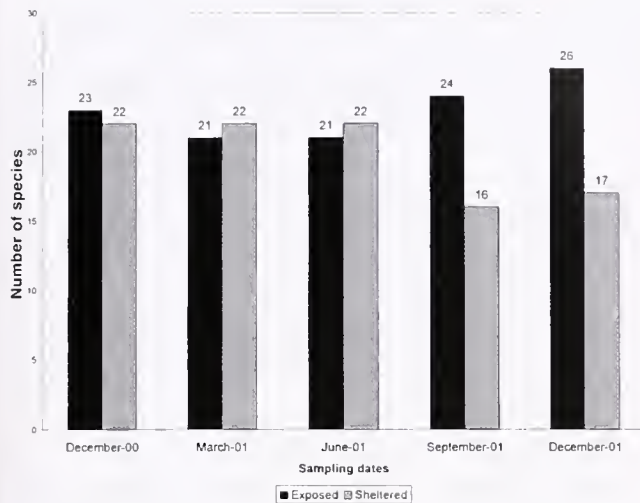


Figure 1. Species richness observed by sampling dates for the exposed and sheltered sites on Isla La Roqueta, December 2000 to December 2001.

dominant species fluctuated per date from 5 in December of 2000 to 10 in December of 2001 (Table 2). The frequency of appearance of the dominant species was distributed in the following manner: nine had 100%, three 80%, three 60% and one 40%.

Of the 9 species that showed 100% frequency of appearance, the gastropod *Plicopurpura patula pansa* (Gould 1853) and the bivalve *Chama echinata* (Broderip 1835) had densities equal to or greater than 1 for all the sampling dates.

The densities of the class Gastropoda for all the dates were higher than those of the classes Bivalvia and Polyplacophora. The highest density of the class Gastropoda was seen in September of 2001, whereas the lowest was recorded in December of 2000. The

class Bivalvia showed the highest density in June and the lowest in September (Table 2).

The Shannon-Wiener index for the annual cycle was 3.165 bits/individual, oscillating among the different samples from 2.432 to 3.250 bits/individual (Fig. 2).

Sheltered Shore

A total of 33 species (Table 1) were found corresponding to 28 genera and 20 families, of which 78.8% belonged to the class Gastropoda, 18.2% to the class Bivalvia and 3.0% to the class Polyplacophora. The least richness was found in the samples of September with 16 species and greatest in December of 2000 and March and June of 2001 with 22 species (Fig. 1).

The mean density during the annual cycle was 88.08 organisms/m², which varied among the different sampling dates from 62.85 to 123.85 organisms/m².

Eleven dominant species were recorded and these species accumulated over the annual cycle to 98.26% dominance. The quantity of dominant species fluctuated per date from seven in December of 2001 to nine in June and September (Table 3). The frequency of appearance of the dominant species was distributed in the following manner: eight had 100%, two 80% and one 40% frequency. Of the 8 that showed 100% frequency of appearance, the gastropods *P. patula pansa*, *Mancinella triangularis* (Bleainville 1832) and *Petalocochus complicatus* Dall, 1908 as well as the bivalves *Chama echinata*, *Brachidontes semilaevis* (Menke 1849) and *Isognomom janus* (Carpenter 1857) had densities equal to or greater than 1 at all the sampling dates.

The densities of the class Gastropoda were higher than those of the classes Bivalvia and Polyplacophora in the samples from June and September, whereas the class Bivalvia showed higher densities than those of the classes Gastropoda and Polyplacophora in samples from December of 2000 and March and December of

TABLE 2.

Densities (organisms/m²) of the dominant species for sampling dates in the exposed site in wave action on Isla La Roqueta, Acapulco, Guerrero México.

Classes/Species	Dec-00	Mar-01	Jun-01	Sep-01	Dec-01
Gastropoda					
<i>Columbella fuscata</i>	0.0	0.0	0.0	1.25	2.45
<i>Crucibulum escutellatum</i>	0.45	0.35	0.2	0.15	2.4
<i>Fissurella geminata</i>	1.15	0.2	0.05	0.3	3.25
<i>Fissurella nigrocincta</i>	0.05	0.0	0.05	2.1	2.1
<i>Hoffmanniella hansi</i>	0.35	0.7	1.4	0.0	0.0
<i>Nodilittorina aspera</i>	0.0	10.95	10.25	30.8	7.0
<i>Nodilittorina modesta</i>	0.10	1.95	0.0	0.0	0.1
<i>Nerita scabricosta</i>	0.0	0.0	0.1	1.55	0.35
<i>Petalocochus complicatus</i>	0.50	24.6	34.15	16.1	20.3
<i>Petalocochus macrophraema</i>	0.10	1.2	6.1	0.25	0.25
<i>Plicopurpura patula pansa</i>	10.10	9.3	10.05	12.85	9.8
<i>Mancinella triangularis</i>	5.7	0.3	2.7	9.0	6.7
Total	18.5	49.55	65.05	74.35	54.7
Bivalvia					
<i>Chama echinata</i>	9.2	3.2	1.75	2.6	8.4
<i>Brachidontes semilaevis</i>	0.0	0.05	17.75	0.65	0.45
<i>Isognomom janus</i>	0.15	0.65	1.7	0.35	0.5
Total	9.35	3.9	21.2	3.6	9.35
Polyplacophora					
<i>Chiton articulatus</i>	1.3	0.95	0.55	0.5	1.85

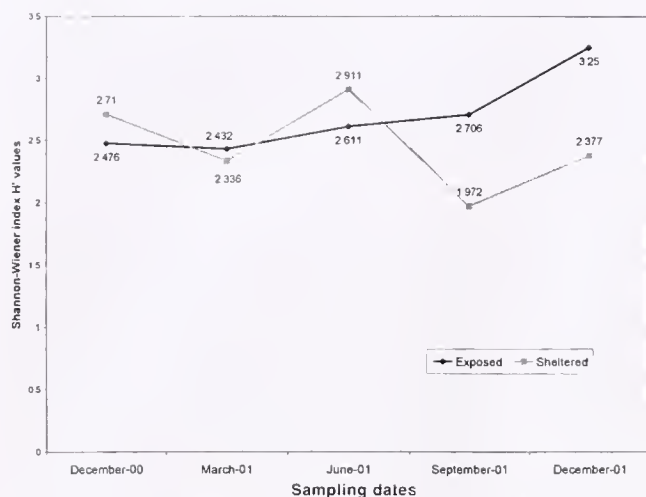


Figure 2. Shannon-Wiener diversity index values, observed for the exposed and sheltered sites on Isla La Roqueta, December 2000 to December 2001.

2001. The highest density for the class Gastropoda was seen in September of 2001, whereas the lowest was recorded in December of 2000. The class Bivalvia showed a higher density in December of 2001 and the lowest in September of the same year (Table 3).

The Shannon-Wiener index for the annual cycle was 2.863 bits/individual, oscillating among the different samples from 1.972 to 2.911 bits/individual (Fig. 2).

Both Sites

Thirty species (68.8%) were common to both sites. Eleven confined species were found at the exposed location, whereas there were only 3 at the sheltered site (Table 1). In the annual cycle, the Jaccard similarity index was 0.682 and the Morisita-Horn quantitative index was 0.622.

The mean number of species found per date was 23 species (SD = 2.12) for the exposed shore and 19.8 species (SD = 3.03) for the sheltered one. The difference between these mean values for

the two sites was not statistically significant (Mann-Whitney = 6.0, $P = 0.222$).

At the exposed site, the mean total density of dominant molluscs of the class Gastropoda for all the samples was 52.43 organisms/m² (SD = 21.24), whereas at the sheltered site it was 44.05 organisms/m² (SD = 33.03). A comparison of the two mean values showed no statistical difference (Mann-Whitney = 9.0, $P = 0.465$). For the class Bivalvia, there were 9.48 organisms/m² (SD = 7.13) at the exposed site and 42.5 organisms/m² (SD = 16.23) on the sheltered shore.

The two locations showed high diversity values, and in comparing the mean Shannon-Wiener indices for the five collections, there were no statistically significant differences (Mann-Whitney = 8.0, $P = 0.421$) (Fig. 2).

DISCUSSION

This study found a greater number species than that reported to date for the upper part of middle intertidal zone of the study area. Villalpando-Canchola (1986) collected 34 species at Isla la Roqueta, in an area referred to as level I, which included the upper part of middle intertidal zone, whereas García-López (1994) found 36 species in the same zone.

The exposed site showed a greater richness, more confined species, a higher number of dominant species, but a lower density. In all the samples, the class Gastropoda showed the greatest density. This site exhibited greater variation with respect to the number of dominant species among the sampling dates, and only two of these species had the characteristic of persisting during the entire cycle as dominant species. These findings indicate that the dominant community showed more mobility among its species.

The sheltered site showed less richness, fewer confined species, a lower number of dominant species, but a higher density. Among the samples, the highest densities per date alternated between the classes Gastropoda and Bivalvia. This location exhibited less variation with respect to number of dominant species among the sampling dates, and six of these species showed the characteristic of remaining the dominant species throughout the entire cycle. These findings indicate that the dominant community had less mobility among its species.

TABLE 3.

Densities (organisms/m²) of the dominant species for sampling dates in the sheltered site to wave action on Isla La Roqueta, Acapulco, Guerrero México.

Classes/Species	Dec-00	Mar-01	Jun-01	Sept-01	Dec-01
Gastropoda					
<i>Columbella fuscata</i>	1.3	0.0	0.05	0.0	0.0
<i>Nodilittorina aspera</i>	0.0	0.55	22.0	79.45	2.35
<i>Nodilittorina modesta</i>	1.0	2.05	7.05	3.35	0.95
<i>Nerita scabricosta</i>	0.0	0.15	1.4	5.05	0.35
<i>Petalconchus complicatus</i>	5.05	7.15	1.15	2.8	6.8
<i>Petalconchus macrophracma</i>	0.75	6.9	0.95	0.45	0.4
<i>Plicopurpura patula pansa</i>	9.0	6.6	7.4	6.7	9.55
<i>Mancinella triangularis</i>	6.1	2.6	5.15	3.15	4.55
Total	23.2	26.0	45.15	100.95	24.95
Bivalvia					
<i>Brachidontes semilaevis</i>	11.4	4.45	15.0	6.05	23.25
<i>Chama equinata</i>	24.5	42.05	20.8	14.25	39.55
<i>Isognomom janus</i>	1.25	1.65	2.75	2.0	3.55
Total	37.15	48.15	38.55	22.3	66.35

Lewis (1964) found a greater richness and more confined species in an exposed site, and Del Río-Zaragoza and Villarroel-Melo (2001) also determined a greater number of species in an exposed site, whereas Spight (1978) reported a greater abundance in a sheltered site. However, García-López (1994) reported a greater number of species at Isla la Roqueta for the sheltered sites studied.

The density of molluscs for the combined dominant species that belonged to the class Gastropoda did not differ between the two locations studied, and gastropods showed the highest abundance of species at both locations. Holguín-Quinones and González-Pedraza (1989), Castillo-Rodríguez and Amezcua-Linares (1992), García-López (1994), and Villarroel et al. (2000) also reported that gastropods displayed the greatest species richness among the classes of molluscs.

On the other hand, the density of molluscs for the combined dominant species belonging to the class Bivalvia did show differences between the two sites, such that the sheltered shore showed a greater number of bivalves.

The molluscs of the class Polyplacophora was recorded as dominant species only at the exposed location.

The percentage of cumulative dominance of the species that had densities equal to or greater than 1 organism/m² was similar for the two sites and was over 98% of the whole malacologic community.

A comparison of the mean values for species richness for all the sampling dates did not show significant differences, indicating that the two locations are similar with regard to richness. This same situation was seen when comparing the mean values for the diversity index H' , which indicates that the sites studied over the annual cycle can be considered equally diverse. However, the values recorded for both richness and the H' index of the sheltered site for each of the samples, demonstrated greater changes in the malaco-

logic community, whereas in the exposed site these values showed more gradual changes.

In this study, the value for the Shannon-Wiener diversity measure for the exposed site was greater when considering the annual cycle. In a study of the same locations where the present work was conducted, García-López (1994) recorded a lower H' index value at the exposed site (1.606 bits/individual), and higher value for the sheltered site (3.817 bits/individual).

The number of common species between the two locations was very high, which was reflected in the value for the Jaccard index. However, García-López (1994) reported a lower index (0.375) for these same locations. According to the Morisita-Horn index, the two sites were quantitatively very similar.

The malacologic community of the upper part of middle intertidal zone of two locations with different exposure to waves was found to be very similar, contrary to the proposed hypothesis of this investigation. However, there are some differences that are important to point out. At the sheltered shore, seasonal changes in richness and diversity were greater despite the lesser variation in its dominant community. At the exposed shore, these changes were more gradual over time despite a greater variation in the dominant community, reflecting a greater stability of the malacologic community at this site.

ACKNOWLEDGMENTS

The authors thank CONACyT for the support offered for the development of this research and Dr. Gorgonio Ruiz Campos for his review of the manuscript and valuable comments. Dr. Enrique Villalpando Canchola, provided invaluable help throughout the study. We appreciate the comments and suggestions from Dr. Emilio Michel Morfín and Dr. Victor Landa Jaime. We also thank Dr. Albert Leyva for his help in the preparation of the manuscript.

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PHYLOGENETIC RELATIONSHIPS AMONG THE DECABRACHIA CEPHALOPODS INFERRED FROM MITOCHONDRIAL DNA SEQUENCES

XIAODONG ZHENG,^{1,*} JIANMIN YANG,^{1,2} XIANGZHI LIN^{1,3} AND RUCAI WANG¹

¹Mariculture Research Laboratory, Fishery College, Ocean University of China, Qingdao, China 266003; ²Marine Fisheries Research Institute of Shandong Province, Yantai, China 264000; ³College of Oceanography and Environmental Sciences, Xiamen University, China 361005

ABSTRACT To clarify phylogenetic relationships among the decabrachia cephalopods, especially the family Sepiidae and Sepiolidae, mitochondrial cytochrome *c* oxidase subunit I (COI) gene and partial 16S rRNA gene were sequenced for 13 species. Phylogenetic analyses were performed by the distance and parsimony method. Coleoids were divided into 2 main lineages, Octobranchia and Decabrachia (including Sepiida, Sepiolida, and Teuthida). In all phylogenetic trees, the monophyly of the Sepiolidae and Sepiidae was supported well, but their rank and position within the Decabrachia were not clear. Based on partial COI rDNA and its amino acid sequences, Parsimony analyses showed Sepiolidae, Sepiidae, and the (*Loligo chinensis*, *Chtenoteryx sicula*) clade from Teuthida were in the same level. Compared with Sepiidae, Sepiolidae was more closely related to Teuthids using 16S rDNA sequences. We are inclined to support the current classification: Sepiolidae and Sepiidae belong to different Orders. According to the phylogenetic analysis, the 2 genera (*Sepiella* and *Sepia*) from the Sepiidae can be distinguished (78% neighbor-joining (NJ); 64% maximum parsimony (MP) in 16S rRNA gene), but do not have visible boundline using COI gene and its amino acid data. This suggests that COI gene may be much fitter to analyze cuttlefish phylogeny at a high taxonomic level (i.e., family), and 16S rRNA gene could be used as a precious tool to analyze taxonomic relationships at the genus level.

KEY WORDS: phylogeny, cephalopods, Decabrachia, COI, 16S rRNA gene

INTRODUCTION

According to Voss (1977), the Sepioidea includes Spirulidae, Sepiidae, Sepiolidae, Idiosepiidae, and Sepiadariidae. To date, the taxon system is still used in China. The position of the Sepiolidae as a sister group of the Sepiidae and the monophyly of the squids are, however, questioned by some scholars (e.g., Berthold & Engeser 1987, Clarke 1988, Boletzky 1999).

Molecular information from DNA data has been used to clarify the relationships among Cephalopods since the 1990s. The nucleotide divergence between sequences of one gene or a portion of gene from the mitochondrial or nuclear genome can be analyzed phylogenetically. Previous DNA sequence diversity and phylogenetic relationships of octopods have been investigated using the mitochondrial cytochrome *c* oxidase subunit I (mtCO I), mtCO II, mtCO III, 16S rRNA gene (Carlini & Graves 1999, Carlini et al. 2001, Bonnaud et al. 1996, Bonnaud et al. 1997, Söller et al. 2000, Allcock & Pietsch 2002, Pietsch et al. 2003). Anderson (2000) sequenced 2 mitochondrial genes (16S rRNA and CO I) to clarify loliginid phylogeny. Sequence analyses from the 3' end of the mt l-rRNA (16S) gene of decapod cephalopods have shown that this portion of gene was a useful tool for taxonomic relationships at the infrafamilial level (Bonnaud et al. 1994). The COI gene for phylogenetic analysis of the coleoid cephalopods exhibited a high degree of nucleotide sequence variability, with one half of the sites varying in at least one taxon; COI amino acid sequences were highly conserved, but were useful in determining basal-level relationships among the Coleoidea (Carlini et al. 2001).

The cephalopods, especially decapods, are an important and valuable fishery resource in China, South Korea, and Japan (Nesis & Kir 1982, Okutani 1995). For example, *Sepia esculenta*, *Sepiella maindroni* and *Loligo chinensis* are all high commercial species (Dong 1991). A great deal of fundamental and applied researches

on cephalopods, such as fauna, systematics, morphology, embryology, population genetics, and biodiversity have been completed since 1960s (e.g., Lee 1963, Lee 1983, Dong 1993, Lu 1998, Lu 2000, Zheng et al. 2001a, Zheng et al. 2001b, Zheng et al. 2004). There are, however, few documents referring to the molecular evolution and phylogenetics of cephalopods living in the coastal waters of China. In this study 13 cephalopod species are analyzed by the sequence comparison of 16S rRNA and COI gene. The phylogenetic trees are reconstructed. The taxonomic relationships of decabrachia species are discussed.

MATERIALS AND METHODS

Taxon Selection

Eight decabrachia species and two octopus species were sampled for 16S rRNA gene sequence analysis. Representatives of three other cephalopods (*Euprymna scolopes*, Bonnaud et al. 1994; *Chtenoteryx sicula*, *Grimpoteuthis* sp., Anderson 2000) were added to cladistic analysis.

Eight decabrachia species and one octopus species were sampled for the COI gene sequence analysis. Representatives of 4 other cephalopods (*Nautilus pompilius*, Carlini & Graves 1999; *Euprymna scolopes*, Bonnaud et al. 1994; *Chtenoteryx sicula*, *Grimpoteuthis* sp. Anderson 2000) were added to cladistic analysis.

Details on the taxonomic position (following Voss 1977) and origin of the 13 species studied are presented in Table 1.

DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted using a CTAB method modified from Winnepeninckx et al. (1993). Regions of the 16S and COI genes were amplified by PCR. The 16S primers sequences were D16SAR 5'-CGC CTG TTT AHY AAA AAC AT-3', D16SBR 5'-CCG GTC TGA ACT CAG MTC AYG T-3' (Anderson 2000). The primers used for the amplification of

*Corresponding author. E-mail: xdzheng@mail.ouc.edu.cn

TABLE 1.
Classification (Voss 1977) of cephalopod taxa included in this study.

Classification	^a References	Locality	GeneCOI/16S#
Phylum MOLLUSCA			
Class CEPHALOPODA			
Subclass NAUTILOIDEA			
Family Nautilidae			
<i>Nautilus pompilius</i> Linne, 1758	CA	Waikiki Aquarium	AF000054/—
Subclass COLEOIDEA			
Order SEPIOIDEA			
Family Sepiidae			
<i>Sepia aculeata</i> Orbigny, 1848	ZH	East China Sea	AF350494/AF369113
<i>S. esculenta</i> Hoyle, 1885	ZH	Yellow Sea of China	AF359554/AF369114
<i>S. latimanus</i> Quoy & Gaimard, 1832	ZH	South China Sea	AY185506/AF369116
<i>S. pharaonis</i> Ehrenberg, 1831	ZH	South China Sea	AF359555/AF369117
<i>S. rohseni</i> (Massy, 1927)	ZH	South China Sea	AF350495/AF369957
<i>Sepiella maindroni</i> de Rochebrune, 1884	ZH	East China Sea	AF340032/AF369118
Family Sepiolidae			
<i>Euprymna berryi</i> Sasaki, 1929	ZH	South China Sea	AF350493/AF369110
<i>E. scolopes</i> Berry, 1913	AN/BO	Hawaii, USA	AF075417/X79592
Order TEUTHOIDEA			
Suborder MYOPSIDA			
Family Loliginidae			
<i>Loligo chinensis</i> Gray, 1849	ZH	South China Sea	AY185505/AF369955
Suborder OEGOPSIDA			
Family Ctenopterygidae			
<i>Ctenopteryx sicula</i> (Verany, 1851)	AN	Pacific Ocean	AF075416/AF110097
Order OCTOPODA			
Suborder CIRRATA			
Family Cirroteuthidae			
<i>Grimpoteuthis</i> sp.	AN	Monterey Bay, CA USA	AF075419/AF110100
Suborder INCIRRATA			
Family Octopodidae			
<i>Octopus ocellatus</i> Gray, 1849	ZH	East China Sea	AF346854/AF369111
<i>O. variabilis</i> (Sasaki, 1929)	ZH	East China Sea	—/AF369112

^a References: AN, Anderson F. E. (2000); CA, Carlini, D. B. and Graves, J. E. (1999); BO, Bonnaud, L. Boucher-Rodoni R, Monnerot M. (1994). ZH, the authors. #Genbank accession numbers.

partial COI gene were: HCO2198 (5'-TAA ACT TGA GGG TGA CCA AAA AAT-3') and LCO1491 (5'-GGT CAA CAA ATC ATA AAG ATA TTG-3') from Folmer et al (1994). Amplifications were performed under the following conditions: 120 s at 94 °C, then 30 or 35 cycles (COI and 16S, respectively) of 40 s at 94 °C, 1 min at 50 °C, and 1 min at 72 °C. A total volume of 25 µL reactions consists of 0.5 units of *Taq* (TaKaRa), 0.5 µM each primer, 0.2 µM each dNTP, 2.5–3.5 mM MgCl₂, 2.5 µl of 10× buffer supplied with *Taq* and 4 µl (30–50 ng) of template DNA.

The amplified fragments were purified by the PCR fragment recovery Kit (TaKaRa). Purified products were sequenced directly using the ABI PRISM BigDye Terminator cycle sequencing Ready Reaction Kit and AmplicTaq DNA polymerase with ABI PRISM 377XL DNA sequencer (Applied Biosystem Inc.).

Phylogenetic Analyses

All of the initial sequences were aligned by ClustalX v 1.8 (Thompson et al. 1997). Amino acid sequences of the COI gene were translated using the GENEDOC (Nicholas et al. 1997). The mean nucleotide composition, proportion of transition (ts) by number of the total base substitutions (ts + tv, transitions + transver-

sions) were calculated in MEGA 2 (Kumar et al. 2001). The phylogenetic analyses were performed on both aligned nucleotide sequences and on amino acid sequences, using distance and parsimony methods included in the packages MEGA 2 (Kumar et al. 2001) and PAUP4.0b6 (Swofford 2000). Statistical confidence of a particular cluster of sequences was evaluated by the bootstrap procedure (1,000 resampling replicates).

RESULTS

Genetic Variation of Partial COI Gene Sequence

The percentage proportion of 4 kinds of base pairs (A, C, G, T) was compared with each other. There was no remarkable difference between these species examined. The average content of base pairs in the nine species examined was 28.12%, 17.51%, 15.71%, and 38.60%, respectively. A + T content was also up to 66%, which is in the same range as in the reference species. High A + T content was an obvious characteristic of cephalopod mtDNA sequences. No gaps were found in all of the sequences we analyzed. A total of 281 nucleotide positions were found to be variable (42.4%). One-hundred and ninety-four variable sites were found at

According to the amino acid sequences, the genetic distance was in the range of 0.000 to 0.0056 within the Sepiidae. *Sepiella maindroni* was grouped with the other cuttlefishes, even though they belong to a different genus in the traditional taxonomy. The farthest distance between *Nautilus pompilius* and other cephalopods (0.119–0.178) indicated that the relationship between them was the most remote.

Genetic Variation of Partial 16S rRNA Gene

In the Sepiidae, the genetic distance of 5 species of *Sepia* ranged from 0.030 to 0.045, and become a bit higher with *Sepiella* (0.059–0.077). The distance between Sepiidae and Sepiolidae (0.085 ± 0.009) was not significantly different from that between Sepiidae and Teuthida (0.090 ± 0.013) or Sepiolidae and Teuthida (0.089 ± 0.012). The longest distance (0.173) was observed between Sepiolidae (*Euprymna scolopes*) and Octopoda (*Octopus variabilis*).

Phylogeny of Cephalopods Based on COI and 16S rRNA Gene Sequence

Phylogenetic Analysis Based on Partial COI and Amino Acid Sequences

The topology structure of phylogenetic trees was similar using the COI and their amino acid sequences, whatever NJ trees and MP trees. The monophyly of the Sepiolidae (98% NJ, 78% MP in Fig. 1; 90% NJ, 74% MP in Fig. 2) and Sepiidae (50% NJ, 58% MP in Fig. 1; 93% NJ, 87% MP in Fig. 2) was well supported. According to NJ analyses, Coleoids were divided into 2 main lineages, Octobranchia and Decabrachia (including Sepiidae, Sepiolidae, and Teuthida). In the Decabrachia clade, 6 species from the Family Sepiidae grouped with 2 species from the Order Teuthida at first, then grouped with the Family Sepiolidae (NJ trees in Fig. 1 and 2), though low levels of bootstrap support were obtained (see asterisk place in Fig. 1 and 2). Parsimony analyses indicated that the Decabrachia was divided into 3 parallel clades (88% MP in Fig. 1; 96% MP in Fig. 2). Within the Sepiidae clade, *Sepiella* (*S. maindroni*) and *Sepia* could not be divided clearly; and the groups from 5 species in *Sepia* were not consistent with morphologic evidence, which belong to 3 different species complexes (Khromov 1998).

Phylogenetic Analysis Based on Partial 16S rRNA Gene Sequence

Both MP and NJ analyses provided strong bootstrap support values for the monophyly of the Coleoid cephalopods (98% MP and 98% NJ), and the monophyly of the Family Sepiidae (64% MP and 78% NJ) as well as Family Sepiolidae (95% MP and 96% NJ) (Fig. 3). Within the Decabrachia clade, Sepiolidae was more closely related with Teuthida (54% MP and 72% NJ). *Sepiella* and *Sepia* were clearly separated (64% MP and 76% NJ). Though the topology structure (NJ tree) in *Sepia* clade was consistent with the classification of species complexes of Sepiidae, bootstrap support values were very low (<50%).

DISCUSSION

Previous investigations of coleoid systematics have attempted to determine relationships within the Sepiida, Octopoda, and Teuthida through phylogenetic analysis of morphologic and molecular character data. Most recently, Carlini and Graves (1999)

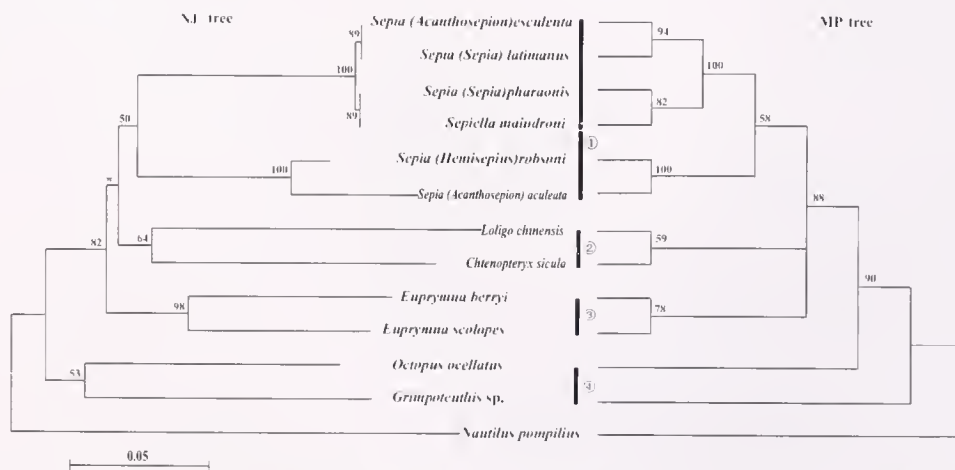


Figure 1. NJ phylogenetic tree and MP tree based on COI gene data. Boldfaced numbers above branches are bootstrap support values (1,000 replicates). Asterisk indicated bootstrap values less than 50%. *Nautilius pompilius* was used as distant outgroup species. (1) Sepiidae, (2) Teuthida, (3) Sepiolidae, (4) Octopodida.

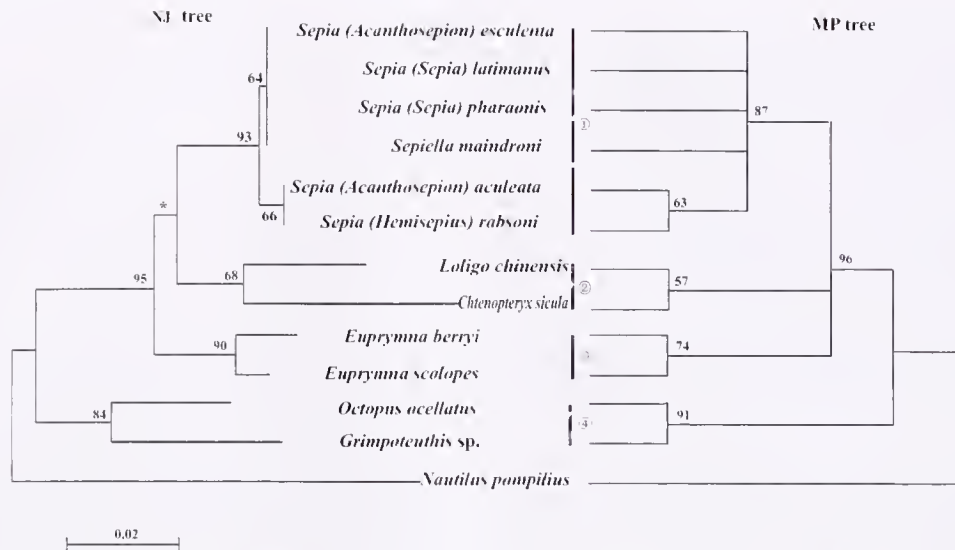


Figure 2. NJ phylogenetic tree and MP tree based on amino acid of COI gene data. Boldfaced numbers above branches are bootstrap support values (1,000 replicates). Asterisk indicated bootstrap values less than 50%. *Nautilus pompilius* was used as distant outgroup species. (1) Sepiidae, (2) Teuthida, (3) Sepiolidae, (4) Octopodida.

conducted a higher-level analysis of the coleoid cephalopods. Consistent with them, our results suggest that the coleoids can be divided into 2 main lineages: the Octobranchia and the Decabrachia.

According to the classification of cephalopod taxa (Voss 1977), the Order Sepioidea was comprised of 5 families Spirulidae, Sepiolidae, Sepiidae, Sepiadariidae, and Idiosepiidae (Fig. 4). Although the COI and 16S rDNA data well supported the monophyly of the Decabrachia, we could not confirm the validity of the order Sepioidea. Several studies have questioned this order (Clarke 1988, Bonnaud et al. 1996, Bonnaud et al. 1997, Sweeney & Roper 1998, Carlini & Graves 1999) (see Fig. 4). Bonnaud et al. (1994) analyzed phylogeny of decapod cephalopods based on 3' end of 16S rDNA nucleotide sequences, and demonstrated that Sepioids were clearly excluded from the order, and the position of the Spirulidae required further clarification. Referring to paleontology and neontology evidences of cephalopods, Clarke (1988) pointed out that the five families belonged to two orders, that is the Order Sepiida was composed of Sepiidae, Spirulidae and Sepia-

dariidae; Sepiolidae and Idiosepiidae belonged to the Order Sepiolida. Sweeney and Roper (1998) listed the currently accepted classification of the recent Cephalopoda and addressed that the five families belonged to three orders. Boletzky (1999) indicated that they should be subject to four orders, and the Superorder Decabrachia included 5 orders. Spirulida, Sepiida, Sepiolida, Idiosepiida, and Teuthida (see Fig. 4). Carlini and Graves (1999) pointed out that the Sepioidea were polyphyletic. In all phylogenetic trees, the monophyly of the Sepiolidae and Sepiidae was supported well, but their rank and position within the Decabrachia were not clear. Based on partial COI and its amino acid sequences, Parsimony analyses showed Sepiolidae, Sepiidae as well as the (*Loligo chinensis*, *Chtenopteryx sicula*) clade from Teuthida were in the same level (88% in Fig. 1 and 96% in Fig. 2). Compared with Sepiidae, Sepiolidae was more closely related to Teuthids (72% NJ and 54% MP) using 16S rDNA. We are inclined to support the current classification: Sepiolidae and Sepiidae belong to different orders. As for the rank and position of other families (e.g., Idi-

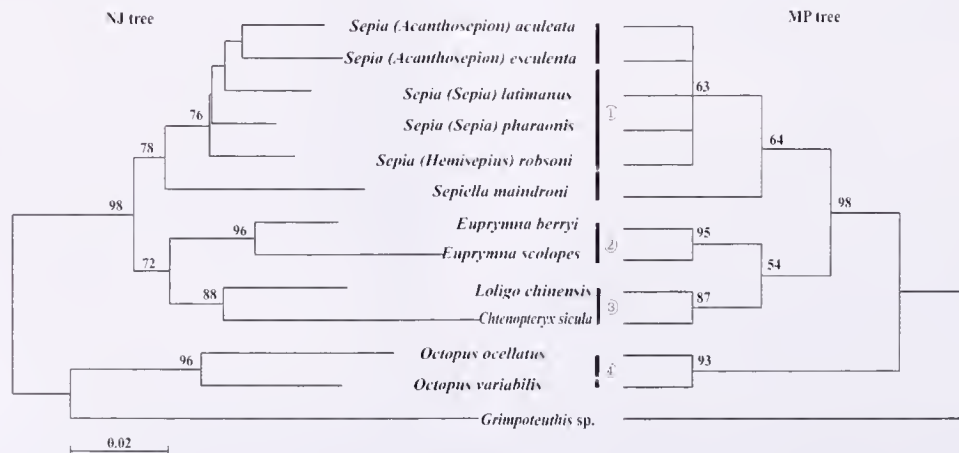


Figure 3. NJ phylogenetic tree and MP tree based on based on 16S rRNA gene data. Boldfaced numbers above branches are bootstrap support values (1000 replicates). *Grimpoteuthis* sp. was used as distant outgroup species. (1) Sepiidae, (2) Sepiolidae, (3) Teuthida, (4) Octopodidae.

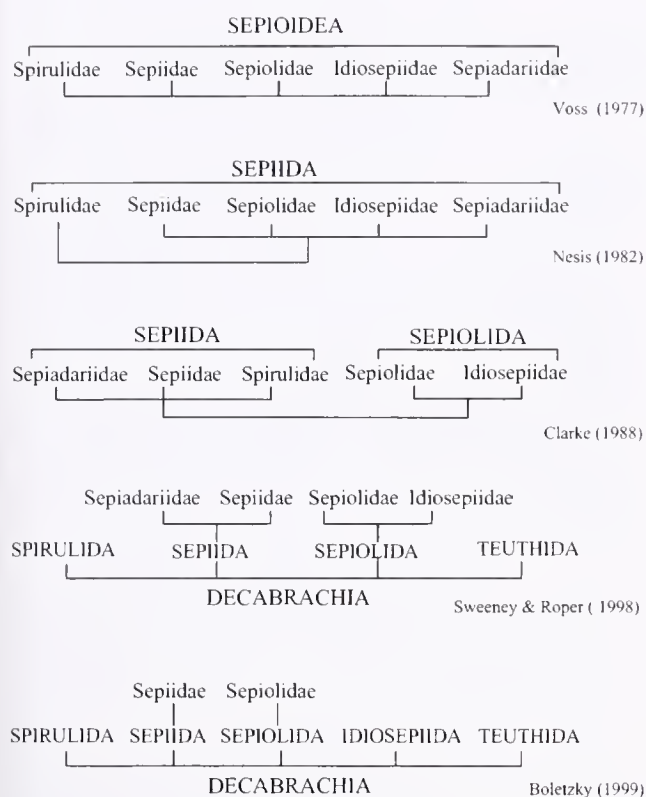


Figure 4. Sepiid relationships with other taxa according to various authors. Ordinal rank indicated by capital letters.

osepiidae, Spirulidae, etc), such researches will be carried out further and testify these families' phylogenetic relationship.

According to the COI gene and amino acid sequence data, the family-level relationship was exhibited clearly, but the genus *Sepiella* (*S. maindroni*) was grouped with other species from *Sepia* (see Fig. 1 and 2). *Sepiella* was obviously separated from the *Sepia* groupings based on the 16S rDNA sequences (see Fig. 3), which demonstrated that they belonged to two separate genera. It shows possibility that mtDNA sequence fragments (e.g., 16S rDNA, COI) with different evolutionary rate are fit for different taxa phylogeny. The 16S rDNA of Cephalopods is a precious tool to analyze taxonomic relationships at the genus level, and COI gene is fitter at the family level. Of course, 3 species complexes within the genus *Sepia* were not well supported in all the MP trees. The recognition of subgeneric rank in *Sepia* is not used because more analyses on morphologic, behavioral, biochemical, and molecular data are needed to resolve the question (Lu 2004, pers. comm.). We will examine more extensive sequence data of the species of *Sepia* for more refined information of species complexes or subgeneric rank.

ACKNOWLEDGMENTS

The authors thank Professor C. C. Lu for valuable suggestions about classification of Cephalopoda, and to Ms. C. W. Ho for help on sequence data analysis. The authors also thank X. D. Du, M. Z. Zeng, W. Z. Chen, and J. M. Zhao, for assistance in the collection of samples, and Ms. Y. L. Wu for helpful comments on the manuscript.

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COMPARISONS BETWEEN THE REPRODUCTIVE BIOLOGY OF FEMALES OF TWO SPECIES OF DEEP SEA CRABS THAT LIVE IN DIFFERENT WATER DEPTHS

KIM D. SMITH, IAN C. POTTER* AND S. ALEX HESP

Centre for Fish and Fisheries Research, School of Biological Sciences and Biotechnology, Division of Science and Engineering, Murdoch University, Murdoch, Western Australia, 6150, Australia

ABSTRACT The reproductive biology of females of *Hypothalassia acerba* and *Chaceon bicolor* off the lower west coast of Australia, and of the former species off the southern coast of Western Australia, are compared. The trends exhibited throughout the year by the prevalence of different stages in ovarian development and of females bearing eggs and egg remnants, together with those of relative gonad weight, demonstrate that the reproduction of *H. acerba* on the lower west coast is highly seasonal. Thus, overall, gonads matured progressively between July and December and oviposition occurred between January and March. The characteristics of *H. acerba* on the south coast differed in the following ways from those on the lower west coast: (1) no ovigerous females and only two females with egg remnants were caught; (2) late yolk granule oocytes were not developed in ovaries until females had reached a larger size; and (3) investment in gonadal development was less. These results strongly indicate that conditions on the south coast are not as conducive for ovarian development and reproduction and that females migrate from the south to lower west coast for spawning. In contrast to *H. acerba*, *C. bicolor* reproduces throughout the year on the lower west coast, presumably reflecting its occupancy of far deeper waters where environmental conditions vary less during the year. Although the mean weights of ovigerous females of *H. acerba* and *C. bicolor* were not significantly different ($P > 0.05$), the mean fecundity of the former species (356,210) was significantly greater ($P < 0.001$) than the latter species (192,070). The greater fecundity of *H. acerba* may reflect adaptations by this seasonal spawner to optimize egg production during its short breeding season.

KEY WORDS: crabs, reproduction, spawning period, water depth, seasonality, gonadal investment

INTRODUCTION

Three species of deep sea crab are fished commercially in Western Australian waters, namely the champagne crab *Hypothalassia acerba* Koh and Ng, the crystal crab *Chaceon bicolor* Manning and Holthuis, and the king crab *Pseudocarcinus gigas* Lamarek (Anonymous 2002). Based on catches obtained by commercial fishers, the distribution of *H. acerba* ranges from a latitude at least as far north as about 27°S on the west coast, southwards, and then eastwards along the south coast to a longitude of at least 129°E (Anonymous 2002). Although *C. bicolor* occurs in the same geographical region as *H. acerba*, its distribution in Western Australia extends further north to a latitude of about 22°S (Melville-Smith et al. in press). In contrast, the distribution of *P. gigas* is essentially restricted to the south coast in Western Australia and extends eastwards across the full length of Australia, including Tasmania, and then northwards on the east coast to about 31°S (Kailola et al. 1993, Gardner 1998).

Recent depth-stratified sampling off the south-western Australian coast has demonstrated that *H. acerba* occurs predominantly in waters where temperatures lie between 13°C and 19°C, the depths range from 200–255 m on the lower west coast, and from 90–200 m on the south coast (Smith et al. 2004a). Temperatures recorded during commercial fishing for *C. bicolor* show that on the lower west coast of Australia this species typically occurs in waters of 4°C to 6.5°C (K. Smith unpublished data) and at water depths of 450–1220 m (Melville-Smith et al. in press); thus this species is located further offshore than *H. acerba*. *Pseudocarcinus gigas* is found in waters of 11°C to 17°C and at depths of 120–340 m (Levings et al. 1996).

The commercial fisheries for *H. acerba* and *C. bicolor* in Western Australia started to develop during the late 1990s (Anonymous 2002). However, in contrast to the situation with *P. gigas* (see Levings et al. 1996, 2001, Gardner 1997, Gardner & Williams

2002), no biological studies have been conducted on the spawning period and location and fecundity of either of these two species.

The stages in development of the ovaries of several species of deep sea crabs belonging to the Geryonidae (e.g., *Chaceon quinquedens*, Smith; *Chaceon maritae*, Manning & Holthuis; and *Chaceon affinis*, Milne Edwards & Bouvier), have been described based on their macroscopic appearance and, in some cases, on the stages of maturation of their oocytes, as determined from histologic sections (Haefner 1977, Melville Smith 1987a, Fernández-Vergaz et al. 2000, López Abellán et al. 2002). No such combined macroscopic and histologic studies have been carried out on any deep sea species of the Eriphiidae, the family to which *H. acerba* belongs. Although some deep sea crab species, such as *Chaceon fenneri* (Manning & Holthuis), have an annual reproductive cycle (see Hastie 1995), no clear seasonal fluctuations in reproductive patterns were detected in *C. maritae* (Melville-Smith 1987a) and *C. quinquedens* (Wigley et al. 1975, Haefner 1978). It was proposed by Melville-Smith (1987a) that the absence of a distinct reproductive cycle in *C. maritae* was attributable to the absence, in the deep waters in which this species is found, of changes in day length and temperature and other potential environmental reproductive triggers.

The aims of this study are as follows. (1) Describe, on the basis of their macroscopic appearance, the sequential stages in the development of the ovaries of *H. acerba* and use histologic sections to determine the stages of maturation of the oocytes in ovaries at each of those stages. Attention is paid to elucidating whether the criteria developed for categorizing the ovarian stages of *H. acerba* were the same as those we developed concomitantly for *C. bicolor* and those described for other species of deep sea crab. (2) Use the trends exhibited throughout the year by the monthly prevalences of female crabs with ovaries at different stages in development and of females bearing either eggs or egg remnants, together with those of the mean monthly gonad weights of females, to determine the timing of reproduction in these two species. Particular emphasis is placed on testing the hypothesis that, as *H. acerba* lives in far

*Corresponding author. E-mail: i.potter@murdoch.edu.au

shallower waters than *C. bicolor*, and thus in an environment which undergoes a greater degree of seasonal change and particularly in water temperature, it is far more likely to reproduce seasonally. (3) Determine the fecundities of *H. acerba* and *C. bicolor* and their relationships to the body size and body weight of these species. (4) Establish whether aspects of the reproductive biology of *H. acerba* on the lower west and southern coasts of Western Australia exhibit similar patterns. Because preliminary analyses indicated that *H. acerba* invests less in gonadal development on the south than west coasts, we examined whether the relative ovarian weights and diameters of the mature oocytes of females on the two coasts differed.

MATERIALS AND METHODS

Collection of Samples

Samples of *Hypothalassia acerba* were randomly selected from trap catches taken by commercial fishers off the lower west and south coasts of Western Australia between ca. 30°10'S, 114°30'E and 32°30'S, 114°60'E and between 34°40'S, and 115°40'E and 34°45'S, 119°30'E, respectively. *Chaceon bicolor* was obtained from the trap catches taken by commercial fishers off the lower west coast of Australia between 24°15'S, 112°15'E and 33°04'S, and 114°23'E. The reader is referred to Smith et al. (2004b) for details of the sampling gear. The samples of *H. acerba* and *C. bicolor*, which were collected at regular intervals between July 1999 and April 2002 and between January 2000 and December 2003, respectively, were pooled with additional smaller samples that were collected by the same methods and purchased regularly from fish markets. This ensured that the monthly prevalences of females at different stages in development and mean monthly relative gonad weights were derived from data for individuals in catches representing, on at least one occasion, each calendar month of the year in the case of each species in the waters in which they were sampled.

Laboratory Processing

The carapace length (CL) and wet weight (W) of each female of *H. acerba* and *C. bicolor* were recorded to the nearest 1 mm and 1 g, respectively. For each female, a record was kept of the relative size and shape of the gonopores and whether mating abrasions were present around their gonopores. Most females of *C. bicolor* with a CL <100 mm possessed elliptical gonopores, indicating that they had not mated, a conclusion consistent with the absence of mating scars in the region of their gonopores (see also Melville-Smith 1987a, Haefner 1977). Although the gonopores of female *H. acerba* did not exhibit such a conspicuous dichotomy in shape as those of *C. bicolor*, the individuals of this species with a CL <ca. 70 mm had relatively very small gonopores and, unlike many larger individuals, they never bore mating scars in the region of their gonopores. The first and small group of *H. acerba* were thus considered not to have mated, which is consistent with the ovaries of all such individuals being at stage I (see Table 1 in results for description of the characteristics of the different stages in ovarian development).

The ovary of each female was removed and allocated to a numerical developmental stage on the basis of macroscopic criteria (see Results). The stages in development of the testes and vas deferentia were recorded during another study aimed at determin-

ing the size at maturity of the males of these two species (Smith et al. in prep.). The ovary of each female of *H. acerba* and *C. bicolor* was weighed to the nearest 0.1 g. The mean monthly ovarian weights ($\pm 95\%$ CIs) at standardized CLs were determined for *H. acerba* on the lower west and south coasts and for *C. bicolor* on the former coast using analysis of covariance (ANCOVA) of the natural logarithm of the gonad weight as the dependant variable, month as a fixed factor and the natural logarithm of the CL as a covariate. Note that the standardized CL was calculated separately for the lower west and south coast assemblages of *H. acerba* and the lower west-coast assemblage of *C. bicolor*. These constant CLs were a default value calculated by the ANCOVA. ANCOVA was also used in the manner (mentioned earlier) to calculate the mean gonad weight ($\pm 95\%$ CIs) for *H. acerba* with ovaries containing previtellogenic, early vitellogenic, and late vitellogenic oocytes on the lower west and south coasts. The standardized CL was calculated separately for each of these three developmental stages, but was common for a given stage on the two coasts. Data for the small individuals that were considered not to have mated were not used when calculating the monthly prevalences of females with ovaries at different stages in development, the mean monthly gonad weights for females at a standardized CL, and the mean gonad weights for west and south coast *H. acerba* with ovaries at different stages in development (see Figs. 2, 3, 4, and 5 in Results).

The method of Melville-Smith (1987a) was used for determining the fecundity of *H. acerba* and *C. bicolor*. Thus, alternate pleopods were removed from the abdomen of each ovigerous female and soaked for 1–4 h in 0.5 M NaOH to facilitate the separation of eggs from the pleopod setae. All of the eggs removed from each crab were pooled, oven dried at 70°C for 24 h and then weighed to the nearest 0.001 g. Subsamples of ca. 0.1 g of eggs (= ca. 300 eggs) were then weighed and counted, and the average weight of a single egg in each subsample was calculated. When estimates of the weight of a single egg in three subsamples differed by less than 5%, the mean of those weight estimates was then used, in conjunction with twice the weight of all eggs removed, to estimate the fecundity of the crab.

The right posterior side of the ovary was removed from up to 20 females of both species in each season, ensuring that these subsamples covered the full size range of those species in the total catch in each season. The ovarian material was fixed in 4% glutaraldehyde in a 0.025 M phosphate buffer solution (pH 7.0), dehydrated in a series of increasing concentrations of ethanols, embedded in paraffin wax, sectioned transversely at 6 μ m, and stained with Mallory's trichrome. The ovarian material was fixed in glutaraldehyde because it was not fixed well by traditional fixatives used for light microscopy, such as Bouin's and 10% formalin. The smallest and largest diameters of oocytes of *H. acerba* and *C. bicolor* at different developmental stages sectioned through their nuclei were measured to the nearest 5 μ m. The smallest and largest diameters of each oocyte were then averaged.

In addition to the earlier mentioned laboratory processing of crabs, the senior author also recorded, when on board fishing vessels, the total number of mature female crabs caught and the percentage of both ovigerous females and of females with egg remnants attached to their pleopods. Note that, due to inclement weather, the commercial fisher with whom we mainly worked did not operate in September in the deep waters off the lower west coast of Australia where *C. bicolor* is found, and thus there are no data on the above variables for that month.

RESULTS

Ovarian Maturation Stages

Based on their macroscopic appearance, the ovaries of the females of *Hypothalassia acerba* and *Chaceon bicolor* each followed essentially the same pattern of development (Table 1). Thus, for both species, six sequential stages were recognized (Table 1; Fig. 1). As they progress from stages I to IV, the ovaries increase progressively in size and change color (see Figs. 1a to d). However, the colors of the ovaries of the two species at stages III and IV differ. In both species, ovaries at stages V and VI are flaccid and could not be separated macroscopically.

At a histologic level, cortical alveolar oocytes first appeared in stage II ovaries of *H. acerba* (see Figs. 1a, b), and the presence of early and late yolk granule oocytes characterized stage III (see Fig. 1c) and IV ovaries (see Fig. 1d), respectively. Ovaries of recently spent females (stage V) of *H. acerba* contained some atretic oocytes (see Fig. 1e), whereas those of spent-recovering (stage VI) females possessed no such oocytes but did contain numerous early previtellogenic oocytes (see Fig. 1f). The histologic characteristics of the ovaries of *C. bicolor* are essentially the same as those shown for *H. acerba* in Figures 1a to f. However, the mean diameters of the mature oocytes, as measured in stage IV ovaries, are significantly greater ($P < 0.001$) for *C. bicolor* than *H. acerba*.

Comparisons of the macroscopic and microscopic characteristics of our ovarian stages for *H. acerba* and *C. bicolor* with those of Haefner (1977) demonstrate that the ovaries of the former two species undergo the same pattern of development as *Chaceon quinquedens*. However, the maximum diameter of the oocytes in our stage I (70 μm) is far less than that recorded by Haefner (1977) for his first stage for *C. quinquedens* (172 μm) and yolk granule oocytes are already present in his stage II whereas they do not appear until stage III of our categorization for *H. acerba* and *C. bicolor*. In essence, Haefner (1977) did not recognize the presence of the early stage we have termed virgin/immature resting. However, that stage was recognized by de Lestang et al. (2003) in their description of ovarian development in *Portunus pelagicus* (Linnaeus) in inshore waters in Western Australia. Furthermore, unlike the study of Haefner (1977), our histologic staging criteria distinguish recently-spent ovaries from spent-recovering ovaries, which is useful for obtaining an idea of how recently ovulation had occurred.

Monthly Trends in Reproductive Indices

On the lower west coast, the prevalence of female *H. acerba* with ovaries containing late vitellogenic oocytes was greatest in September to February, during which months their contributions ranged from 58% to 76%, and was least in March to June when

TABLE 1.

Macroscopic and microscopic characteristics of sequential stages in ovarian development of *Hypothalassia acerba* and *Chaceon bicolor*. Range in oocyte diameters at each stage of development are provided. Scheme has been adapted from that of Haefner (1977).

Ovary Stage	Macroscopic Description	Histological Description
Stage I Virgin/immature resting	Ovaries small but generally visible macroscopically, thin and 'H' shaped. (Shape is maintained for all latter stages except stage IV). Oocytes not visible through ovarian wall. Color varies from pale grey to ivory.	Ovaries contain oogonia and small previtellogenic oocytes (chromatin nucleolar and perinucleolar oocytes). Connective tissue and oocytes stain blue with Mallory's trichrome. For <i>H. acerba</i> , oogonia 5–10 μm and small previtellogenic oocytes 10–60 μm . For <i>C. bicolor</i> , oogonia 5–10 μm and small previtellogenic oocytes 10–70 μm .
Stage II Developing	Ovaries always visible macroscopically, larger than stage I and slightly dorso-ventrally compressed. Ovaries beginning to displace hepatopancreas. Oocytes not visible through ovarian wall. Color varies from ivory to white.	Ovaries contain all oocyte stages present in stage I together with cortical alveolar oocytes. Cortical alveolar oocytes 60–200 μm in <i>H. acerba</i> , 70–210 μm in <i>C. bicolor</i> . Cortical alveolar oocytes stain blue with Mallory's trichrome, except for their lipid droplets and nucleolei, which stain dark red.
Stage III Maturing	Ovaries larger than at stage II and outer membrane has wrinkled appearance. Oocytes not visible through ovarian wall. Ovaries are pale pink in <i>H. acerba</i> , but range from very pale yellow to yellowish orange in <i>C. bicolor</i> .	Large numbers of early yolk granule oocytes present. Yolk granules in peripheral region of oocyte cytoplasm. Yolk granules stain orange with Mallory's trichrome and are larger than lipid droplets. For <i>H. acerba</i> , yolk granule oocytes are 160–270 μm and for <i>C. bicolor</i> are 140–390 μm .
Stage IV Mature	Ovaries larger than at stage III and consequently are no longer 'H-shaped'. Ovarian wall more folded. Oocytes visible through ovarian wall. Ovaries pink to red in <i>H. acerba</i> and brown to purple in <i>C. bicolor</i> .	Large numbers of late yolk granule oocytes. Yolk granules distributed throughout cytoplasm. For <i>H. acerba</i> yolk granule oocytes are 200–480 μm and for <i>C. bicolor</i> 350–560 μm .
Stage V Recently spent	Ovaries similar in appearance to stage II, but more flaccid and yellowish-grey in colour.	Ovary contains mainly connective tissue. Some yolk granule oocytes present and undergoing atresia.
Stage VI Spent-recovering	Same as stage V.	Ovaries comprise mainly connective tissue and numerous small previtellogenic oocytes.

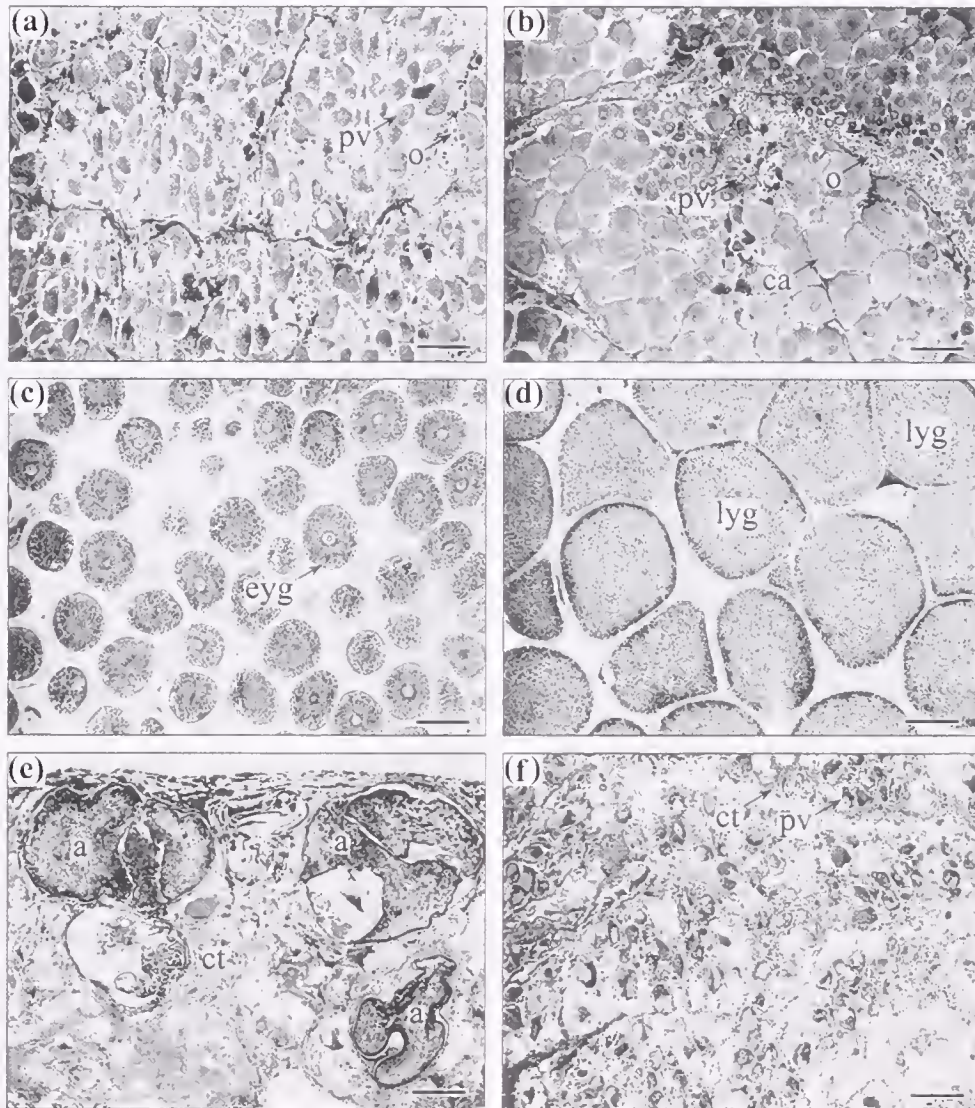


Figure 1. Histologic sections of ovaries of *H. acerba* at (a) stage I, (b) stage II, (c) stage III, (d) stage IV, (e) stage V, and (f) stage VI. a, atretic oocyte; ca, cortical alveolar oocyte; ct, connective tissue; eyg, early yolk granule; o, oogonia; lyg, late yolk granule; pv, previtellogenic oocyte. Scale bar = 200 μ m.

their monthly contributions ranged from 11% to 50% (Fig. 2a). The prevalence of female crabs with ovaries containing only previtellogenic oocytes was less than 12% in each month from September to February and in April and only exceeded 30% in March and August. Small numbers of spent females were collected between January and March and in May. The mean monthly gonad weight of *H. acerba*, standardized to a constant CL of 94.7 mm, rose progressively from 11.5 g in July to between 25.2 and 28.6 g in October to December, and then it declined sequentially to between 4.9 and 8.3 g in March to May (see Fig. 2b). The prevalence of female *H. acerba* bearing egg remnants declined sequentially from 12% in July to zero or close to zero in October to January before rising progressively to 21% in May. Small numbers of ovigerous females were caught in February, March, and May (see Fig. 2c) and a commercial fisher provided six ovigerous females and two females with egg remnants, which he had caught in January 2000.

On the south coast, the prevalence of female *H. acerba* containing late vitellogenic oocytes and the mean monthly gonad

weights, standardized to a common CL of 95.2 mm, peaked in September (Figs. 3a, b). The prevalence of *H. acerba* with ovaries containing late vitellogenic oocytes never exceeded 47% in any month other than September and the prevalence of ovaries with previtellogenic oocytes exceeded 50% in 5 months (see Fig. 3a). Samples from the south coast yielded no ovigerous females and only two females bearing egg remnants, both of which were caught in August.

The prevalences of females of *C. bicolor* with ovaries containing late vitellogenic oocytes were greater in July to December than in January to April and in June (Fig. 4a). The mean monthly gonad weights, standardized to a constant CL of 110.0 mm, peaked at 42.2 g in September and 43.1 g in December before declining to 11.6 g in February, increasing to 37.1 g in May and then declining to 8.8 g in June (see Fig. 4b). Ovigerous females were caught in each of the 11 calendar months in which samples were obtained and females bearing egg remnants were found in 6 of those months (see Fig. 4c). The mean gonad weight \pm 95% CIs, standardized to a constant CL, did not differ significantly ($P > 0.05$) between the

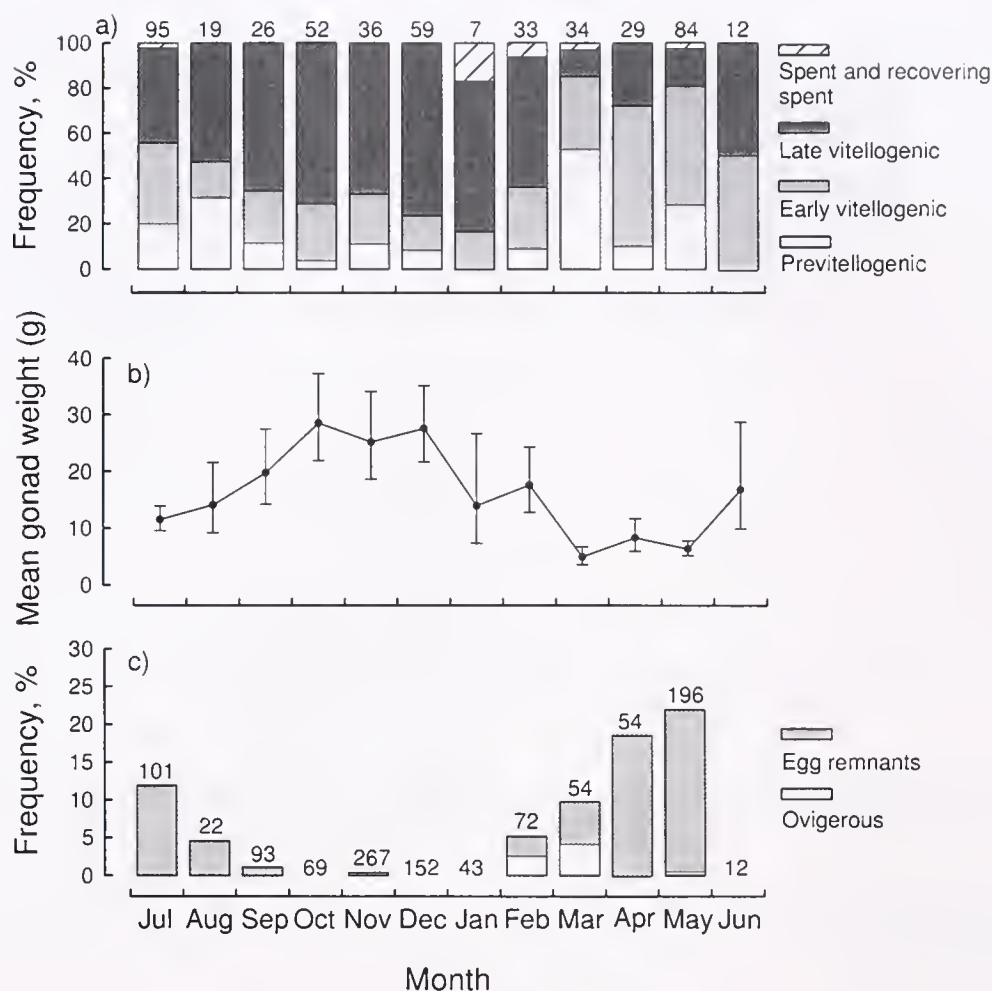


Figure 2. Monthly data for female *Hypothalassia acerba* on the lower west coast of Australia. (a) Frequency of occurrence of crabs at different stages of development, (b) mean gonad weights $\pm 95\%$ CIs standardized to a constant CL of 94.7 mm, as selected by ANCOVA and (c) frequency of occurrence of crabs with eggs and egg remnants. In this Figure and Figures 3 and 4, the sample sizes for each month in (b) are the same as those given in (a).

assemblages on the lower west and south coasts when the females contained ovaries with either previtellogenic or early vitellogenic eggs (Fig. 5). In contrast, the mean gonad weight $\pm 95\%$ CIs for females of *H. acerba* with ovaries containing late vitellogenic oocytes was significantly greater ($P < 0.001$) for the assemblage on the lower west coast, 26.4 ± 1.71 g, than that on the south coast, 16.0 ± 1.85 g (see Fig. 5).

The relationships between gonad W and CL of west coast and south coast females of *H. acerba* with ovaries containing late vitellogenic oocytes (Fig. 6) are described by the following equations.

West coast: $\ln(W) = 2.561\ln(CL) - 8.130$; $P < 0.001$, $R^2 = 0.240$, $n = 228$

South coast: $\ln(W) = 6.430\ln(CL) - 26.311$; $P < 0.001$, $R^2 = 0.442$, $n = 34$

Although none of the females of *H. acerba* from the south coast, which had CL s < 90 mm, contained ovaries with late vitellogenic oocytes, many females of *H. acerba* from the lower west coast with CL s of 75 to 90 mm possessed ovaries with oocytes at this advanced stage. The use of ANCOVA demonstrated that, in

the case of *H. acerba* for a standardized selected CL , the mean gonad weight of crabs was significantly greater on the lower west coast than south coast ($P < 0.001$).

To enable predictions of CL from W and vice versa for *H. acerba*, the regression equations relating these 2 variables are:

West coast: $\ln(W) = 2.809\ln(CL) - 6.400$; $P < 0.001$, $R^2 = 0.946$, $n = 581$

South coast: $\ln(W) = 2.768\ln(CL) - 6.128$; $P < 0.001$, $R^2 = 0.893$, $n = 185$

The mean lengths ($\pm 95\%$ CIs) of females from the lower west (93.8 ± 2.02 mm) and south coasts (93.0 ± 1.39 mm) were not significantly different ($P > 0.05$) when the individuals contained previtellogenic oocytes, whereas the corresponding values of 94.7 ± 0.91 mm and 99.1 ± 1.37 mm for females with late vitellogenic oocytes on those two coasts were significantly different ($P < 0.001$). These differences are reflected in the fact that the distributions of the CL s of female *H. acerba* on the south coast were located more to the right than those on the lower west coast in the case of females with late vitellogenic oocytes, whereas this was not so with females with previtellogenic oocytes (Fig. 7a, b).

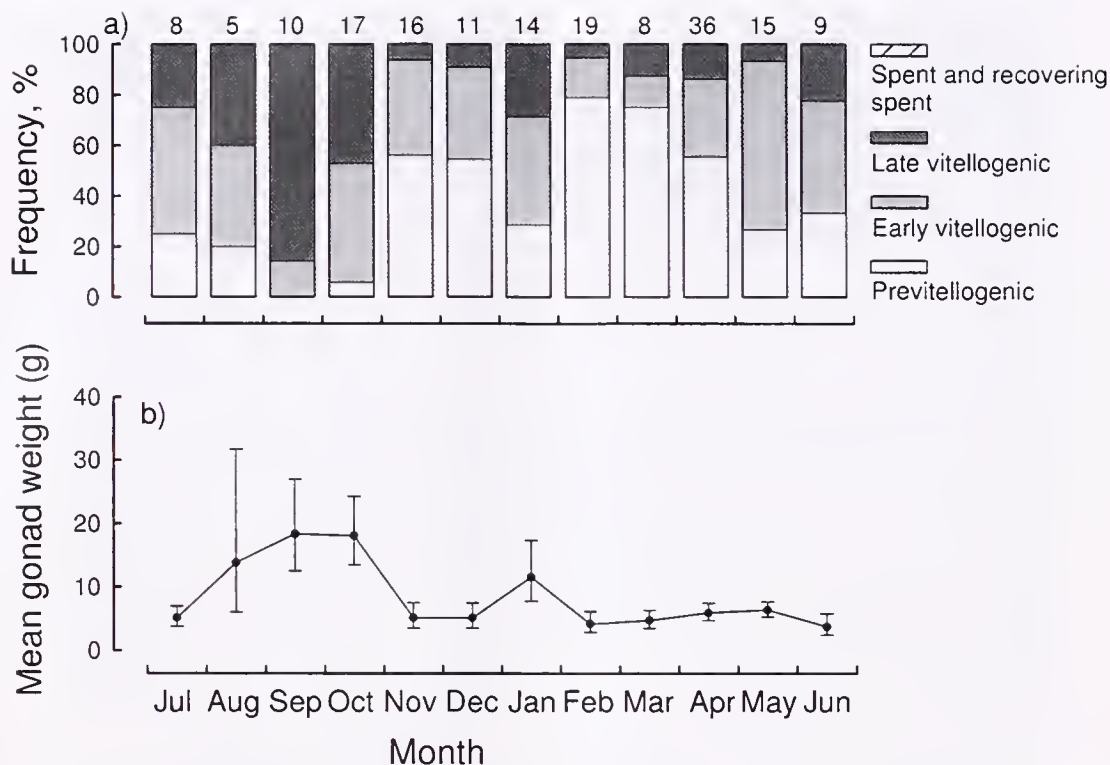


Figure 3. Monthly data for female *Hypothalassia acerba* on the south coast of Australia. (a) Frequency of occurrence of crabs at different stages of development and (b) mean gonad weights ± 95 CIs standardized to a constant CL of 95.2 mm, as selected by ANCOVA.

The mean diameters ($\pm 95\%$ CIs) of late vitellogenic oocytes of *H. acerba* from the lower west coast ($357.7 \pm 10.79 \mu\text{m}$, $n = 342$) and south coast ($370.3 \pm 8.16 \mu\text{m}$, $n = 134$) were not significantly different ($P > 0.05$).

The fecundities of *H. acerba* ranged from 202,295 to 488,758 for crabs with CLs of 90 mm and 102 mm, respectively. The minimum and maximum fecundities for *C. bicolor* were 15,592 and 288,512 respectively, for crabs with CLs of 98 and 133 mm respectively. The mean fecundities ($\pm 95\%$ CIs) of *H. acerba* (i.e., $356,210 [\pm 64,297]$) and *C. bicolor* (i.e., $192,070 [\pm 33,640]$), were significantly different ($P < 0.001$). Although the mean body weights ($\pm 95\%$ CIs) of the ovigerous females of *H. acerba* used for the above fecundity estimates (i.e., $587.4 [\pm 96.5 \text{ g}]$) were substantially less than that of *C. bicolor* (i.e., $651.9 [\pm 61.3 \text{ g}]$), those means were not significantly different ($P > 0.05$). The relationships between fecundity (F) and the CL and wet W of the two species are shown in Figures 8a to d and are described by the following regression equations:

Hypothalassia acerba

$$\ln F = 3.44 \ln(CL) + 1.235, P < 0.05, R^2 = 0.486, n = 10$$

$$F = 0.005W + 0.608, P < 0.05, R^2 = 0.569, n = 10$$

Chaceon bicolor

$$\ln F = 2.957 \ln(CL) + 1.686, P < 0.05, R^2 = 0.127, n = 40$$

$$F = 0.003W + 0.1572, P < 0.05, R^2 = 0.260, n = 40$$

DISCUSSION

Comparisons Between Hypothalassia acerba on the West and South Coasts

The trends exhibited by the three reproductive variables recorded for the females of *Hypothalassia acerba* on the lower west coast of Australia were consistent with each other and clearly imply that reproduction in this deep sea species is highly seasonal. Thus, the increase in the prevalence of females with ovaries containing late vitellogenic oocytes between July and October and subsequent leveling off to December was paralleled by a progressive rise and then leveling off in the mean monthly relative gonad weights. Likewise, the subsequent decline in the prevalence of females with this category of advanced ovary between December and March was accompanied by a pronounced decline in the mean monthly relative gonad weights. Furthermore, spent females were found in the short period of January to March, which yielded most of the ovigerous females obtained during this study. It is also highly relevant that the prevalence of female *H. acerba* bearing egg remnants rose progressively from zero or low levels in October to January to peak in May and subsequently declined sequentially to zero between July and October. The consistent trends exhibited by the monthly data for the three reproductive variables strongly suggest that oviposition occurs mainly in January to March.

The patterns exhibited by the reproductive variables for female *H. acerba* on the south coast of Western Australia differed markedly from those displayed by this species on the lower west coast. Thus, on the south coast, the prevalence of females with late vi-

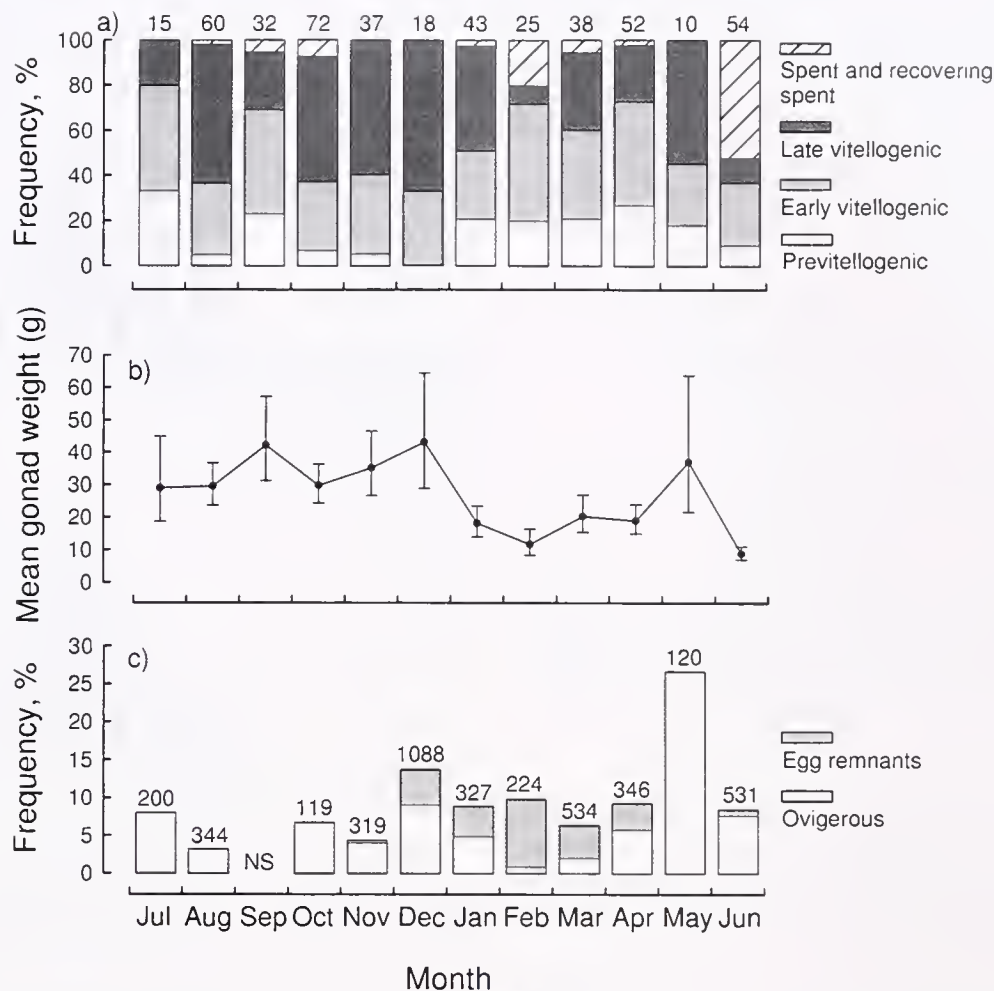


Figure 4. Monthly data for female *Chaceon bicolor* on the lower west coast of Australia. (a) Frequency of occurrence of crabs at different stages of development and (b) mean gonad weights $\pm 95\%$ CIs standardized to a constant CL of 110.0 mm, as selected by ANCOVA and (c) frequency of occurrence of crabs with eggs and egg remnants. NS, not sampled.

tellogenic oocytes and the mean monthly relative gonad weights both peaked in September rather than December and the overall prevalence of females with ovaries containing previtellogenic oocytes was far higher. Moreover, no ovigerous female crabs and only two females with egg remnants were collected on the south coast. This strongly suggests that very little reproduction occurs on the south coast.

The possibility that the lack of capture of ovigerous females of *H. acerba* on the south coast is due to females moving inshore prior to oviposition, as occurs, for example, with *Chaceon affinis*, *Chaceon quinquedens*, *Chaceon maritae*, and *Chionoectes opilio* (Fabricus) (Haefner 1978, Melville-Smith 1987a, Comeau et al. 1998, Hastie 1995, López Abellán et al. 2002) seems unlikely. This conclusion is based on the results of our stratified sampling in sequential water depth intervals from 35 m to 365 m, which showed that the females and males of *H. acerba* were both largely concentrated in a relatively narrow range of water depths of 200–255 m off the lower west coast and of 90–200 m off the south coast, and that no crabs were caught in water depths of 35 m (Smith et al. 2004a). However, it is relevant that, although the catches of *H. acerba* on both coasts contained a greater number of

males than females, that difference was substantially greater on the south coast (Smith et al. 2004a). The earlier mentioned data on *H. acerba* suggest that, at some stage prior to oviposition, the females of *H. acerba* on the south coast typically migrate eastwards and then northwards on the lower west coast where they extrude their eggs.

The conclusion that the females of large and maturing *H. acerba* undergo considerable migratory movements is consistent with the observation that such movements are undertaken, in particular, by mature females of some other species of deep sea crabs (e.g., *Chaceon fenneri*, *C. quinquedens*, and *C. maritae* [Melville-Smith 1987b, Lockhart et al. 1990]). Some species of lobster also move substantial distances, including the western rock lobster *Panulirus cygnus* George, along the west coast of Australia (Phillips 1983, Melville-Smith & Cheng 2002). In decapods, these types of movements are often related to reproduction and enable those species to return to their spawning locations as mature adults after they have been transported as larvae or juveniles away from the areas of spawning (e.g., Bennett & Brown 1983, Moore & MacFarlane 1984, Stewart & Kennelly 1988, Groeneveld & Branch 2002). In the context of possible larval movement in south-

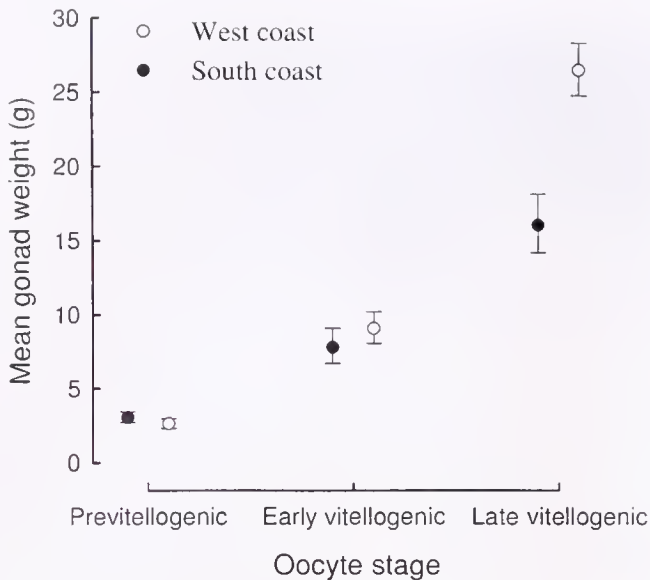


Figure 5. Mean gonad weights $\pm 95\%$ CIs for female *Hypothalassia acerba* caught off the lower west versus south coasts and containing ovaries with predominantly previtellogenic, early vitellogenic and late vitellogenic oocytes. The gonad weights of each of the ovarian categories on both coasts were standardized to constant CL as selected by ANCOVA.

western Australia, it is relevant that the important Leeuwin current flows southwards along the lower west coast of Australia and then eastwards along the south coast and extends to a depth of 350 m (Pearce & Griffiths 1991). It is thus proposed that the larvae of *H. acerba* are planktonic, as has been shown by Gardner (1998) to be the case with those of the confamilial *Pseudocarcinus gigas*, and would thus be likely to be transported by this major current from the areas on the lower west coast where spawning occurs. The likelihood of such transport would be enhanced by the fact that the

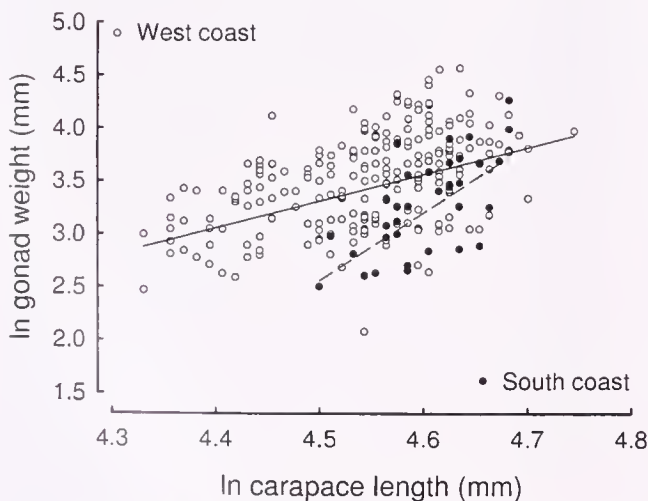


Figure 6. Relationships between the weights of ovaries containing late vitellogenic oocytes and CL for *Hypothalassia acerba* from both the lower west and south coasts of Western Australia.

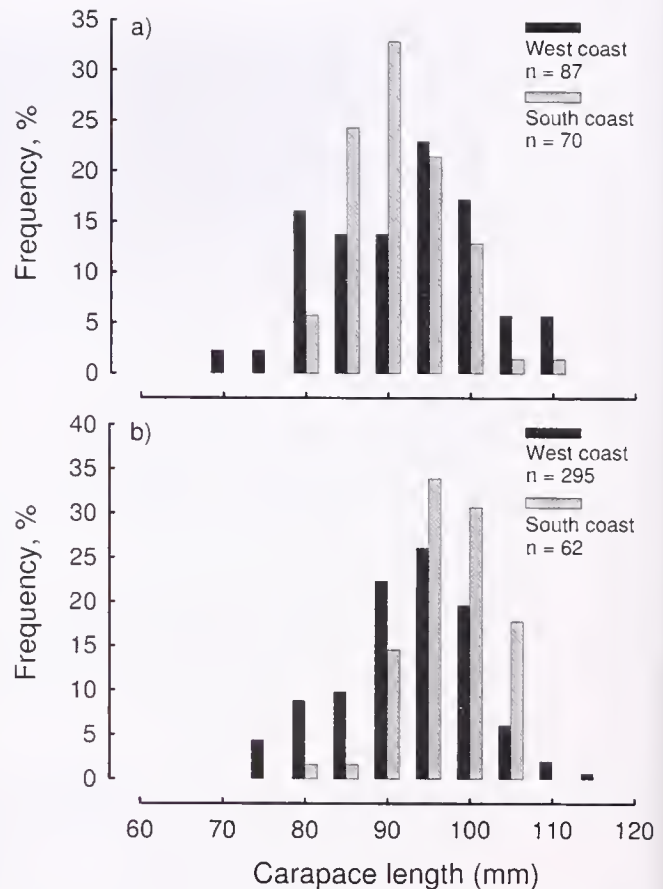


Figure 7. CL-frequency distributions for female *Hypothalassia acerba* with ovaries containing (a) previtellogenic and (b) late vitellogenic oocytes off the lower west and south coasts of Western Australia.

larvae of *H. acerba* on the west coast are released in autumn, when the Leeuwin current is strongest (Phillips et al. 1991).

Comparisons Between *Hypothalassia acerba* and *Chaceon bicolor*

The trends exhibited by the reproductive variables for *Chaceon bicolor* were far less seasonal than those displayed by *H. acerba*. Thus, although the prevalence of females of *C. bicolor* with ovaries containing late vitellogenic oocytes was high in October to December, it was also relatively high in August and May and, whereas the mean monthly relative gonad weight declined between December and February, it remained high from July to December. More importantly, however, *C. bicolor* with spent/spent-recovering ovaries were caught in 8 months and in each season of the year, thereby indicating that oviposition occurs in most months of the year. This conclusion is consistent with the fact that ovigerous females of *C. bicolor* were caught in each of the 11 months for which samples were obtained for deriving this variable and that, unlike the situation with *H. acerba*, the prevalence of such females did not exhibit a clear pattern of change during the year. The finding that, unlike the situation with *H. acerba* and *P. gigas*, which live in similar water depths (Levings et al. 1996), *C. bicolor* clearly does not reproduce within a restricted part of the year and is consistent with *C. bicolor* occupying far greater depths and thus living in an environment that undergoes little seasonal change. The

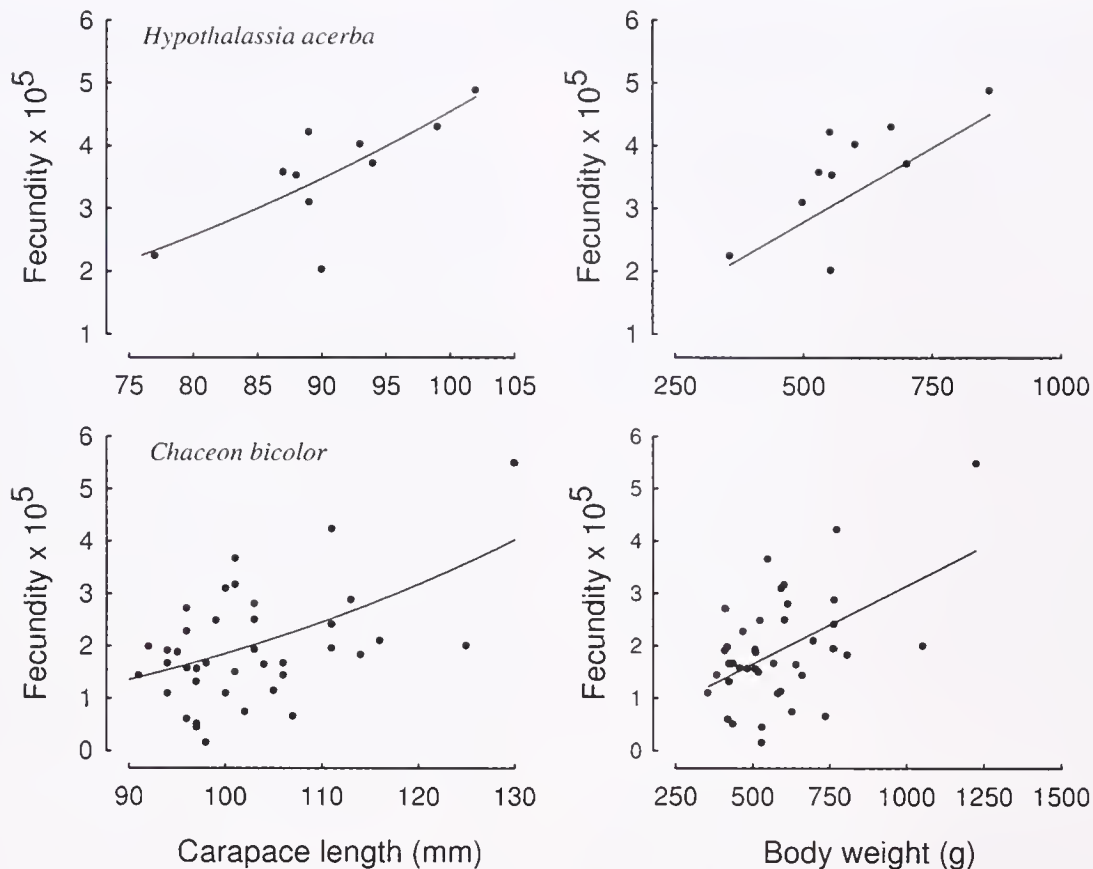


Figure 8. Relationships between fecundity and CL and body weight for *Hypothalassia acerba* and *Chaceon bicolor*.

lack of conspicuous seasonality in the reproduction of *C. bicolor* parallels that found by Melville-Smith (1987a) for *Chaceon maritae*, which likewise lives in deep water. It is also consistent with the fact that the physiologic processes of the majority of organisms living in deep waters do not exhibit seasonality (Tyler 1988).

Investment in Gonadal Development by *Hypothalassia acerba*

It was particularly noteworthy that the mean relative gonad weights of females with late vitellogenic oocytes were far greater for the assemblage on the west coast than for that on the south coast, especially as this did not apply to females with oocytes in earlier stages of development. This strongly indicates that the females of *H. acerba* on the west coast invest less energy in ovarian development than those on the south coast. Furthermore, the lack of a significant difference between the size of the late vitellogenic oocytes of females on the south and lower west coasts implies that any differences in energy allocation to ovarian development on the two coasts is not reflected in differences in an allocation to individual oocytes. From the earlier mentioned data and comparisons, it seems reasonable also to assume that the number of eggs per batch produced by females of *H. acerba* that originated on the south coast are likely to be less than those of the individuals that spent at least most of their life on the west coast.

It is also evident from the data shown in Figure 6 that the females of *H. acerba* on the south coast do not start producing

mature (late vitellogenic) oocytes until they have reached a substantially larger size than those on the west coast. This implies that, in terms of size, the attainment of maturity is delayed on the south coast compared with the west coast.

In summary, our results provide good circumstantial evidence that conditions are less conducive for gonadal development and reproduction by *H. acerba* on the south than lower west coast of Western Australia. Furthermore, they also suggest that maturing females of *H. acerba* migrate from the south coast, which is at the southern and cool end of the distribution of this species, to the west coast for spawning. Reproduction on the lower west coast was shown to occur seasonally in *H. acerba*, but throughout at least much of the year with *C. bicolor*, which occurs in far deeper water and thus where environmental conditions remain more constant during the year. The seasonality of reproduction by *H. acerba* is accompanied by producing, at any one time, a greater number of eggs per given body weight than does *C. bicolor*.

ACKNOWLEDGMENTS

The authors thank fishers B. Maguire, C. Neave, G. Pateman, G. Wilson, R. Prior, and T. Goodall for help with collecting samples, S. de Lestang for helpful discussion, the Australian Fisheries Research and Development Corporation, and Murdoch University for financial support.

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A SURVEY OF GENETIC CHANGES AND SEARCH FOR SEX-SPECIFIC MARKERS BY AFLP AND SAMPL IN A BREEDING PROGRAM OF CHINESE SHRIMP (*PENAEUS CHINENSIS*)

LIUSUO ZHANG,¹ XIAOYU KONG,¹ ZINIU YU,^{1*} JIE KONG,² AND LIMEI CHEN¹

¹The Key Laboratory of Mariculture of Ministry of Education, Ocean University of China, Qingdao 266003, People's Republic of China and ²Yellow Sea Institute of Fisheries Research, Chinese Academy of Fisheries Science, Qingdao 266071, People's Republic of China

ABSTRACT Amplified fragment length polymorphism (AFLP) and selective amplified microsatellite polymorphic loci (SAMPL) were used for a survey of genetic changes over three generations and search for sex-specific markers in a breeding program of Chinese shrimp *Penaeus chinensis*. For genetic survey, a total of 247 and 140 clearly defined bands from 6 AFLP and 4 SAMPL primer sets, respectively, were generated. Both estimated percentage of polymorphic loci (ranging from 41.3% to 47.8%) and average gene diversity (ranging from 0.168 to 0.190) were not significantly different from each other among generation samples, suggesting no significant change at level of genetic variation by AFLP and SAMPL analysis. Despite the frequency change of band-presence allele at all loci over generations showed no clear and traceable patterns, the values of genetic distances and identities between founder stock and sequential generations reflected the accumulation of genetic changes over generations, probably due to isolation and selection. Analysis of molecular variance (AMOVA) and pairwise Φ_{PT} statistics of AFLP and SAMPL data indicated that significant genetic variation was distributed among generation samples ($P < 0.01$). The use of more variable markers may help further examination of genetic change over generations in more details. Search for sex-specific markers with AFLP and SAMPL loci in this study failed to detect any putative markers, despite 2,110 bands in total were generated through extensive screening with 25 AFLP and 16 SAMPL primer pairs. The inability in detection of sex-specific markers may be due to any of several reasons such as weak correlation between the genotypic and phenotypic sex, high genetic diversity in sex-related regions, or just lack of enough number of loci for screening.

KEY WORDS: shrimp, *Penaeus chinensis*, AFLP, SAMPL, genetic changes, sex-specific markers

INTRODUCTION

The Chinese shrimp, *Penaeus chinensis*, has been an important species for the shrimp fishery for many decades in China (Yu & Chan 1986). Its aquaculture has developed rapidly since the success of artificial propagation in early 1980s and the production reached 200,000 tons per year in the early 1990s (Wang et al. 1997). Unfortunately, the extensive outbreaks of shrimp diseases (mainly white spot syndrome virus, WSSV) in 1993, and the following few years resulted in significant decline in production in those intensive production areas. The development of disease-resistant strains of Chinese shrimp represents a major task for the scientific and aquaculture community.

A selection program aimed to develop disease resistance (mainly to WSSV) strains was initiated in 1998, and mass selection has been conducted for three generations (a higher survival rate than that in control was observed). For a breeding program, it is usually important that not only the phenotypic performance of the selected strain be closely checked, but also genetic changes over generations be monitored carefully. A major concern for selected populations is possible loss of genetic variability and inbreeding (Allendorf & Ryman 1987, Hedgecock & Sly 1990, Hedgecock et al. 1992, Gaffney et al. 1992). Loss of genetic variation reduces response to selection and severe inbreeding may lead to poor survival and slow growth (Virjenhoek et al. 1990, Launey & Hedgecock 2001).

Shrimp still remains among the list of aquatic animals in which little is known about sex differentiation and determination. Although significant difference in growth rate exists between female and male, as with many other species, no sex chromosomes has

been detected yet (Xiang et al. 1993), and no sex-specific genetic marker has been reported in *P. chinensis*.

Advances in molecular biology have provided various polymorphism techniques for study of genetic variation and search for genetic markers linked to specific traits. Amplified fragment length polymorphism (AFLP, Vos et al. 1995) is a popular polymorphism system that combines the advantages of RFLP and PCR, with no requirement of prior sequence characterization of the target genome. The number of polymorphisms detected and the level of fingerprint reproducibility per reaction is much higher than that revealed by most of the other PCR-based methods because of its simultaneous coverage of many loci in a single assay. Selective amplified microsatellite polymorphic loci (SAMPL) analysis (Witsenboer et al. 1997) is a modification of AFLP methodology, which utilizes the same template DNA as that of AFLP. However, the selective amplification uses one of the AFLP primers in combination with a specially designed SAMPL primer. The SAMPL primer essentially comprises of a compound microsatellite sequence, and such a SAMPL primer design ensures preferential amplification of microsatellite-like sequences.

In this study, AFLP and SAMPL have been used for analyzing genetic changes over generations of a selected strain of *P. chinensis*. At the same time, a search for sex-specific markers was conducted for potential identification of markers associated with sex differentiation and determination, using these two marker systems.

MATERIALS AND METHODS

Sample Collection and DNA Isolation

Thirty-six individuals representing four generations sample (9 from each generation randomly) were obtained from Yellow Sea Institute of Fisheries Research, Chinese Academy of Fisheries Sci-

*Corresponding author. E-mail: carlzyu@ouc.edu.cn

ences. Four generations were designated as G_0 , G_1 , G_2 , and G_3 , respectively. G_0 sample was the founder stock for selection, which was the progeny of several hundreds of wild shrimp captured in Weihai waters, the Yellow Sea, China. Mass selection was conducted with disease challenge for 3 generations (G_1 through G_3). Briefly, about 200 mature females and males (roughly equal number of each sex) from G_0 were used to reproduce in a local hatchery. These females with an attached spermatophore were separated for egg release and fertilization to build the next generation (G_1). Juvenile shrimp were grown in local shrimp ponds until maturation. One year later, about the same number of females and males were selected from G_1 and mated to establish G_2 in the same hatchery. The same work was repeated for the establishment of the G_3 population.

Genomic DNA was extracted from muscle using the method described by Aljanabi and Martinez (1997). DNA samples were checked by agarose gel electrophoresis, and their concentrations were determined with Beckman-spectrophotometer (Model DU 520, Beckman). For search of sex-specific markers, two separate pools consisting of DNAs from four females and five males, respectively, were used to minimize possible effects of individual variation and improve efficiency of screening process.

AFLP Analysis

AFLP procedure was performed as described by Vos et al. (1995). Genomic DNAs were digested with *EcoRI* and *MseI*, and ligated with relevant adaptors overnight at room temperature. Pre-selective amplification was performed using primers with one selective base at their 3' end (E-A and M-C). For genetic survey, 6 pairs of selective primers, each containing 3 selective nucleotides at their 3' end, were used for selective PCR. The 6 selective primer pairs were E-AAG/M-CCT, E-AAG/M-CTG, E-AAG/M-CAC, E-AAG/M-CGA, E-ACG/M-CTC, and E-ACG/M-CCT, respectively. For the search of sex-specific markers, 25 AFLP (5 *EcoRI* primers \times 5 *MseI* primers) primer pairs were used. The 5 *EcoRI* primers included E-AAC, AAG, ACA, ACC, and ACG, and 5 *MseI* primers were M-CAC, CCT, CGA, CTC, and CTG, respectively.

With the addition of an equal volume of sequencing dye (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue), PCR products obtained in the selective reaction were denatured at 95 °C for 5 min, and then immediately cooled on ice for at least 5 min. A 2.5 μ L of each sample was loaded on a denaturing polyacrylamide (6%) gel and run at 60 W. After silver staining (Bassam et al. 1991), bands were visualized under white-illumination. The developed gels were dried at room temperature for 24 h before scoring the bands.

SAMPL Analysis

SAMPL procedures used here were the same as that of AFLP, described earlier in the section of AFLP Analysis, except that SAMPL primers replaced *MseI* primers in the selective amplifications (Witsenboer et al. 1997). For genetic survey, 4 selective primer pairs were used, which included E-AAG/SAM01, E-AAG/SAM02, E-ACG/SAM01, and E-ACG/SAM02, respectively (Table 1). For the search of sex-specific markers, 16 (3 *EcoRI* and 1 *MseI* primers \times 4 SAMPL primers) primer pairs were used. They were E-AAC, AAG, ACG and M-CGA, and 4 SAMPL primers, respectively (see Table 1).

TABLE 1.

Sequences of the four SAMPL primers used in this study.

Primer	Sequence
SAM01:	5'-KKVRVRVCTCTCTCTCT-3'
SAM02:	5'-KKVRVRVCTCTCTCTCTC-3'
SAM03:	5'-KKRYRYRACACACACAC-3'
SAM04:	5'-KKHVHVHTGTGTGTGTG-3'

K = G or C; R = A or G; Y = C or T; H = A or C or T; V = A or C or G.

Data Analysis

AFLP and SAMPL profiles were scored manually as "1"s (band presence) or "0"s (band absence). Two assumptions were made: (1) bands come from independent nuclear loci and do not migrate to the same position on gel; and (2) each band represents a dominant genotype at a locus, where lack of the same band in another individual correspond to the alternative homozygous recessive genotype in the Hardy-Weinberg equilibrium (Lynch & Milligan 1994).

The unbiased estimator of Lynch and Milligan (1994) was used to estimate frequency of two alleles (1 and 0) at each locus and expected heterozygosity with combined AFLP and SAMPL data was calculated accordingly. Pairwise genetic distances and genetic identities were calculated with two data sets jointly using POPGENE (Yeh et al. 1999). AMOVA and pairwise Φ_{PT} statistics of AFLP and SAMPL data were conducted to determine genetic difference of the generations with GenAlEx V5 (Peakall & Smouse 2001).

RESULTS

In genetic survey, six AFLP primer combinations and four SAMPL primer pairs generated 247 and 140 clearly defined bands, respectively, averaging 41.2 bands per primer pair for AFLP and 35.0 bands for SAMPL. Most amplified profiles were highly specific and reproducible, and ambiguous bands were excluded from further analysis. The size of most scored bands ranged from 100 to 400 bp. The percentage of polymorphic loci and average gene diversity (Nei et al. 1975) with combined AFLP and SAMPL data ranged from 41.3 to 47.8% and from 0.168 to 0.190, respectively (Table 2), which were not significantly different from each other (Chi-test, $P = 0.56$ and 0.99 , respectively), suggesting no significant change in the level of genetic variation by AFLP and SAMPL. The frequency change of band-presence allele at all loci over generations showed no clear and traceable patterns.

The genetic distances and genetic identities with combined data sets among generations are shown in Table 3. Clearly, the values of distances or identities between G_0 and other generations increased or decreased over successive order accordingly, reflecting the accumulation of genetic changes over generations. AMOVA

TABLE 2.

Average percentage of polymorphic loci and Nei's (1973) gene diversity with combined AFLP and SAMPL data.

	G_0	G_1	G_2	G_3
Percentage of polymorphic loci	41.3	45.7	43.4	47.8
Nei's gene diversity	0.168	0.183	0.173	0.190

TABLE 3.

Nei's (1978) genetic distances (below diagonal) and genetic identities (above diagonal) among generations with joint AFLP and SAMPL data.

	G ₀	G ₁	G ₂	G ₃
G ₀	—	0.9187	0.8797	0.8652
G ₁	0.0848	—	0.9415	0.9161
G ₂	0.1282	0.0603	—	0.9294
G ₃	0.1448	0.0876	0.0732	—

results from both data sets showed that significant genetic variation was distributed among generation samples ($P < 0.01$), with percentages of total variance of 17% and 26%, respectively, among generations for AFLP and SAMPL data (Table 4). Furthermore, pairwise Φ_{PT} statistics in both data sets indicate that significant genetic difference occurred between any neighbor generation pair and increased over sequential generation order (Table 5).

With respect to the search of sex-specific marker, 2,110 bands in total were generated with AFLP and SAMPL, but unfortunately, analysis and comparison showed that no putative sex-specific band was identified for both sexes in the two marker systems.

DISCUSSION

Genetic Changes over Generations

While AFLP has been extensively used in the studies of genetic diversity of populations, germplasms, cultivars, and strains for plants, it has recently been applied to similar researches in aquatic animals (Seki et al. 1999, Moore et al. 1999, Miller et al. 2000, David et al. 2001). AFLP is considered very efficient at revealing genetic difference due to its ability to screen polymorphism on genome scale despite lower average heterozygosity (Vos et al. 1995, Maguire et al. 2002). SAMPL is a novel approach that emerged recently for genetic studies and shown useful in plants. Although based on small sample sizes, the similar estimation of average percentage of polymorphic loci and gene diversity of four samples indicated that no significant change at level of genetic variation was observed among generations by AFLP and SAMPL analysis. This maintenance of genetic variation level is likely due to the large size of founder population and the reproduction populations used in the following generations. It could help reduce the adverse effects of genetic drift, selection, and inbreeding that may occur in a breeding program. This result should be contributed by the fact that AFLP's advantage of comprehensive evaluation of multi polymorphic loci on genome range compensated the effect of

the small sample size. With AFLP, Seki et al. (1999) detected significant and clear genetic difference among three Ayu *Plecoglossus altivelis* populations (8 fish per sample) by using 19 primer combinations; and significant difference in average heterozygosity for three samples were also found. Similarly, it was confirmed that multilocus RAPD (6 random primers) detected very similar molecular differentiation among blacklip abalone *Haliotis rubra* populations in small sample size (10 animals per sample) as revealed by microsatellites (Huang et al. 2000).

Usually, heterozygosity level and allele diversity shift are considered as indicators for genetic changes of populations under selection or culture. Sbordoni et al. (1986) examined genetic diversity with 20 allozyme loci for sequential hatchery generations in *P. japonicus*, noticing progressive reduction of heterozygosity from 0.102 down to 0.039 in samples from generation F₁ through F₆. However, Cruz et al. (2004) found that high heterozygosities (in 2 microsatellite loci) were still maintained through two generations of the Pacific white shrimp *Litopenaeus vannamei* under selection. Nevertheless, three studies of penaeids species have reported lower numbers of alleles at microsatellite loci in cultured populations than in wild or founder stocks (Wolfus et al. 1997, Bierne et al. 2000, Xu et al. 2001). In molluscan, similarly, several studies showed that no association was observed between selection or culture and loss of heterozygosity in the Pacific oyster *Crassostrea gigas* (Hedgecock & Sly 1990), hard clam *Mercenaria mercenaria* (Dillon & Manzi 1987) and American oyster *C. virginica* (Vrijenhoek et al. 1990, Yu & Guo 2004). However, the reduction of allele number in these selected populations was commonly observed (Dillon & Manzi 1987, Vrijenhoek et al. 1990, Yu & Guo 2004). Obviously, loss of genetic variation resulted from genetic drift, bottlenecks, selection, and inbreeding caused by a reduced effective population size.

Despite no significant change in the level of genetic variation was observed through AFLP and SAMPL analysis, it is risky to say that no significant change at level of genetic variation occurred at all. Because allele diversity change, which is another indicator of genetic changes, could not be detected via these two methods. From the literature mentioned above, one can see that the two indicators do not match in many cases. Therefore, for more details of genetic change over generations, further studies using microsatellite or allozyme markers will be helpful and necessary. Recently, some microsatellites have been developed for Chinese shrimp (Liu et al. 2004) and could be used for further analysis of this breeding program.

Because isolation and selection of stocks may lead to deterioration via inbreeding and reduction of effective population size, genetic changes should be monitored carefully following operation of selection. Despite complicated frequency change of band-presence allele and unavailability of traceable patterns of polymorphic loci over generations in this study, AFLP and SAMPL

TABLE 4.

Analysis of molecular variance of AFLP and SAMPL in 4 generations.

Data	Source of Variation	df	Variance	% Total	Φ Statistics	P Value
AFLP	Among generations	3	4.66	17	0.165	0.001
	Within generations	32	23.54	83		
SAMPL	Among generations	3	3.85	26	0.260	0.001
	Within generations	32	10.96	74		

TABLE 5.

Pairwise Φ_{PT} statistics of AFLP (below diagonal) and SAMPL (above diagonal) in generations. P value in parenthesis.

	G ₀	G ₁	G ₂	G ₃
G ₀	—	0.281 (0.001)	0.406 (0.001)	0.410 (0.001)
G ₁	0.118 (0.001)	—	0.066 (0.067)	0.182 (0.002)
G ₂	0.202 (0.001)	0.106 (0.011)	—	0.135 (0.005)
G ₃	0.257 (0.001)	0.177 (0.005)	0.106 (0.005)	—

did screen out occurrence of difference over generation samples. As indicated by genetic distances, identities and result of AMOVA, isolation and selection caused the accumulation of genetic changes over sequential generations.

From AMOVA of SAMPL data, it was noted that estimation of percentage of total variance distributed among generations was higher than that in AFLP data (26% vs. 17%), so was from pairwise Φ_{PT} statistics of SAMPL. This difference was very likely due to the characters of polymorphism from SAMPL. Theoretically, SAMPL screens 4 types of polymorphisms: (1) codominant microsatellite polymorphisms, which resulted from the number variation of repeat units within microsatellites targeted by the microsatellite-anchor primers; (2) presence/absence-style polymorphisms arising from variation of the annealing sites for the microsatellite-anchor primer; (3) codominant polymorphisms originating from insertion/deletions in amplified fragments; and (4) presence/absence-style polymorphisms originating from variation in restriction sites (Yang et al. 2001). Whereas type 2 and 4 could be detected as dominant polymorphism, type 1 and 3 may cause incorrect scoring information due to their codominant nature, and then magnified the difference among samples involved to some extent. In addition, another reason responsible for this difference could be the smaller data size (140 bands) in SAMPL than that in AFLP (247 bands).

Search for Sex-specific Markers

Understanding the mechanism of sex determination and differentiation is important for study of shrimp biology and aquaculture practices. Although recently Li et al. (2003) mapped sex-linked markers on the maternal linkage map in Kuruma prawn *P. japonicus* and implied that the female may be the heterogametic sex in this species, search for sex-specific markers with AFLP and SAMPL loci in current study has failed to detect any putative

markers, despite extensive screening with 25 AFLP and 16 SAMPL primer pairs (2,110 bands in total were generated). Previously, attempts to detect sex-specific markers in Atlantic salmon (*Salmo salar*) and green spotted pufferfish (*Tetraodon nigroviridis*) with RAPD, AFLP, and RDA (representational difference analysis) were made, but were not successful (McGowan & Davidson 1998, Li et al. 2002). Sex determination in crustacean has been studied for the last 2 decades (Malecha 1983, Legrand et al. 1987, Benzie 1998, Hulata 2001), with the exception of decapods. Lack of sex chromosome differentiation in penaeids has been observed, and no environmental factors of sex determination have ever been reported (Korpelainen 1990). As mentioned earlier, karyotype analysis detected no dimorphic pair of chromosomes, and it may imply absence of sex chromosomes or their weak differentiation in *P. chinensis* genome. The inability to detect of sex-specific markers in this kind of limited search could be due to any of the following reasons: very weak correlation between the genotypic and phenotypic sex due to modifiers, high genetic diversity in sex-related regions on genome among the individuals studied, or biallelic and autosomal nature of sex-determining genes (Li et al. 2002, McGowan & Davidson 1998). Of course, it could not be ruled out that the number of loci used in this search may not be enough to screen out the sex-specific markers, if there is any. More work is needed before we come to any conclusion.

ACKNOWLEDGMENTS

The authors thank Mr. B.W. Liu and D. R. Hu for their assistance with DNA extraction and Mr. Sean Boyd for English review. This work was supported by an 863 project of Ministry of Science and Technology of China (2001AA620105) and funded by the Key Laboratory of Mariculture of Ministry of Education, Ocean University of China.

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EFFECTS OF TEMPERATURE AND SALINITY FLUCTUATION ON THE OXYGEN CONSUMPTION, AMMONIUM EXCRETION AND OSMOREGULATION OF THE BLUE SHRIMP *LITOPENAEUS STYLIROSTRIS* (STIMPSON)

FERNANDO DÍAZ,^{1,*} ANA DENISSE RE,¹ ELIZABETH SIERRA¹ AND EUGENIO DÍAZ-IGLESIAS²

¹Departamento de Biotecnología Marina, Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Km. 107, Carretera Tijuana-Ensenada, Ensenada Baja California, México and

²Departamento de Acuicultura, Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Km 107, Carretera Tijuana-Ensenada, Ensenada Baja California, México

ABSTRACT This study presents data on the effect of temperature and fluctuations in salinity on the oxygen consumption, nitrogen excretion, and osmoregulation of *Litopenaeus stylirostris* juveniles to determine the combinations, so these can be used to optimize the culture conditions. The oxygen consumption rate of juveniles acclimated to 23°C, 28°C, and 33°C was measured. Fluctuating salinity levels were applied to these organisms in a sequence of 40‰, 32‰, 25‰, 16‰, 25‰, 32‰, and back to 40‰. The results indicate that metabolic rate of blue shrimp exposed to salinity fluctuation was not affected significantly. When the salinity was reduced from 40‰ to 25‰ the ammonium excretion of the shrimp was increased, in an 16‰ salinity in all temperatures the ammonium production increased in average of 220%, when the salinity was increased from 16‰ to 40‰ the ammonium excretion of the shrimp was reduced. Hemolymph osmolality of the organisms was hyposmotic as the salinity was reduced from 40‰ to 33‰, hyperosmotic during the 25‰ to 16‰ interval and hyposmotic as the salinity increased from 25‰ to 40‰. The isosmotic points had an interval of 676.8–700.7 mmol Kg⁻¹ (23.7‰ to 24.6‰). The excretion rate of ammonium of the shrimp acclimated at temperatures of 23°C, 28°C, and 33°C was related with the pattern of osmoregulation, when the shrimp were hyper-regulators, ammonium increased and diminished when they were hypo-regulators. To optimize the culture of *L. stylirostris* in controlled conditions, we propose that they be cultivated in salinities of 25‰ and temperatures of 28°C because these are considered the optimal conditions, also this environment is free of stress, and for this reason the growth of shrimp is increased.

KEY WORDS: shrimp, temperature, salinity fluctuation, oxygen consumption, ammonium excretion, osmoregulation, *Litopenaeus stylirostris*

INTRODUCTION

Temperature and salinity of the water are among the most important environmental factors that affect the life of the penaeids shrimp (Chen & Nan 1995). The adaptative capacity of the penaeids is specific and determined by a number of factors, that have caused shrimp species to be distributed differently in the marine-estuarine gradient (Claybrook 1983). In recent years the studies on the physiology of the penaeids have increased, due to interest in the aquacultural practice with these organisms; through these investigations optimal conditions for their cultivation can be delimited (Brito et al. 2000).

One of the physiologic responses that can be correlated with the changes of the environmental parameters is the oxygen consumption rate, because it is related with the metabolic work and energy flow that the organisms must destine for the mechanisms of homeostatic control (Salvato et al. 2001). Therefore the measurement of the oxygen consumption in the aquatic animals is a valid method to evaluate the effect of environmental factors such as temperature, salinity, exposition to pollutants, light intensity, and the dissolved oxygen, because it allows the determination of the energetic cost associated with the physiologic stress that these combinations impose on the organisms (Kinne 1970, Villarreal & Rivera 1993, Brown & Terwilliger 1999, Lemos et al. 2001, Altinok & Grizzle 2003).

The ammonium excretion rate has been used to evaluate the effect of various environmental factors on the physiology of the crustaceans (Jiang et al. 2000). The ammonium represents 40% to 90% of the total nitrogen excreted by the crustaceans and is con-

tinuously released through the branchial epithelium (Hartenstein 1970, Kinne 1977, Regnault 1987). A decrease of the environmental osmotic concentration gives a result of a decrease on the amino acids in the tissues and an increase in the ammonium excretion on the organisms (Lange 1972). Significant effects of temperature and salinity on ammonium excretion have been quantified in some species of crustaceans of commercial importance as in *Macrobrachium rosenbergii*, De Man (Nelson et al. 1977, Stern et al. 1984, Díaz Herrera & Buckle Ramirez 1993); *Fenneropenaeus indicus*, Milne-Edwards (Gerhardt 1980); *F. chinensis*, Osbeck (Chen & Lin 1992, 1995); *Marsupenaeus japonicus*, Bate (Chen & Lai 1993); *Penaeus monodon*, Fabricius (Chen et al. 1994); *Farfantepenaeus aztecus*, Ives (Hernández & Díaz 1995); and *Litopenaeus vannamei*, Boone (Jiang et al. 2000, Díaz et al. 2001).

The environmental variation of the salinity disorganizes the osmotic balance in the penaeids, provoking the readjustment of the osmotic concentration of the hemolymph on these organisms. The ability for osmoregulation in decapods was evaluated by Charman-tier et al. (1988, 1989) through the osmoregulatory capacity (OC), defined as the difference between the osmolality of the hemolymph and the external medium osmolality for a given salinity. The OC is a useful tool to evaluate the physiologic condition of the shrimps as well as to detect the sublethal effects of the stress in the culture systems (Lignot et al. 2000).

During the last 4 decades, the osmoregulatory physiology of different species of penaeids has been studied to evaluate the optimal conditions for their culture as in: *Farfantepenaeus aztecus* (Bishop et al. 1980, Castille & Lawrence 1981, Howe et al. 1982, Hernández & Díaz 1995); in *Litopenaeus setiferus*, Linnaeus *L. stylirostris* Stimpson and *L. vannamei* (Williams 1960, Castille & Lawrence 1981, Rodríguez 1981); in *P. esculentus*, Haswell; *Fen-*

*Corresponding author. E-mail: fdiaz@cicese.mx

neropenaeus merguensis, De Man; and *Metapenaeus bennettiae* Racek and Dall (Dall 1981); in *F. indicus* (Parado-Esteva et al. 1987); in *P. monodon* (Cawthorne et al. 1983, Ferraris et al. 1986); in *Marsupenaeus japonicus* (Charmantier-Daures et al. 1989); in *Fenneropenaeus chinensis* (Chen & Lin 1994, Chen et al. 1995, Chen & Lin 1998); and *P. semisulcatus*, De Hann (Clark 1992). However, the osmoregulation processes in these organisms were studied in regimes of constant salinity, whereas in the lagoon-estuarine systems in which these crustaceans normally inhabit, the factors are unstable and present marked variations in temperature, salinity, and dissolved oxygen throughout the year (Salvato et al. 2001).

The blue shrimp *Litopenaeus stylirostris* is distributed from Punta Abrejos, Baja California down to Tumbes, Peru. This species is commercially cultivated in Ecuador and México, inhabits lagoons, estuaries and bays, places with a varying hydrography through the year, affecting organisms that inhabit in these aquatic systems, due to well defined periods of rain and drought (low water) (Rodríguez 1981, Aragón 2000). The aquacultural potential that the blue shrimp *L. stylirostris* has in México makes it necessary to know the effect of the variations of temperature and salinity on the oxygen consumption, nitrogen excretion and the osmoregulatory capacity, to determine the adequate environmental conditions to optimize its cultivation.

MATERIALS AND METHODS

A number of *Litopenaeus stylirostris* juveniles ($n = 1500$) was acquired in the production farm "Camarón Dorado S.A." located in Huatabampo, Sonora, México. The organisms were placed on three reservoirs of 2000 l in the laboratory with continuous flow of seawater and constant aeration. They were maintained at a temperature of $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and a salinity of 35‰ during a week. The shrimps ($n = 900$) with a wet weight of 2.8 to 5.0 g were transferred to three circular reservoirs of 500 l for acclimation during 30 days to the temperatures of 23°C , 28°C , and $33^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and 35‰ salinity. The temperatures of 23°C and 33°C were obtained by decreasing or increasing the temperature of the water of the reservoirs at a rate of 2°C by day. During this phase the shrimps were fed twice a day with Rangen diet for shrimp with 35% of protein. Once the period of thermal acclimation was over, salinity was increased to $40\text{‰} \pm 1\text{‰}$ and remained in these conditions for 6 days, sufficient time for the shrimps to stabilize their internal medium (Bishop et al. 1980, Rosas et al. 2001).

Of each experimental condition, 20 organisms in intermoult stage were selected. The stage of the moult cycle was identified according to the procedure described by Huner and Colvin (1979), 12 h before initiating the measurements they were introduced individually in a semi-opened respirometer as described by Díaz et al. (1989) which consists of 21 respirometric chambers of 1 l. These organisms, as well as the remainders ($n = 140$) that stayed in the reservoirs, were exposed during 4 h in a cycle of 28 h simulating a natural variation similar to an estuarine system, to the effect of the salinity fluctuation as the salinities were changed in sequence to 40‰, 32‰, 25‰, 16‰, 25‰, 32‰, and 40‰, according to the methodology described by Vanegas et al. (1988). To obtain the salinities lower than 35‰ dilutions were carried out using freshwater and the salinity of 40‰ was obtained by the addition of Instant Ocean salts. The shrimps were not fed 24 h before beginning the determination of oxygen consumption, ammonium excretion, and osmoregulatory capacity.

The measurements of oxygen consumption and ammonium excretion were quantified simultaneously in 20 organisms placed in the respirometric chambers, the water flow on the chambers remained open for 3 hours to let the adjustment of the internal medium of the organisms exposed to a change of salinity (Díaz et al. 2001). Before closing the flow, two water samples were taken to measure the initial oxygen and ammonium concentration. Subsequently the chambers remained closed for 1 hour to avoid a reduction of the dissolved oxygen of 25% to 30%, and this constituted a stress factor (Stern et al. 1984). Before the water flow was re-established in the chambers, two water samples were taken to measure the final dissolved oxygen and ammonium production.

The dissolved oxygen concentration in the water samples were measured with a dissolved oxygen meter YSI, model 52, provided with a polarographic sensor, which was inside of an acrylic hermetic chamber with a 10-mL capacity with adequate stirring. The rate of oxygen consumption of the shrimps was calculated as the difference between the initial and the final concentrations and it was expressed as $\text{mg O}_2 \text{ h}^{-1} \text{ g}^{-1}$ of dry weight (d.w.) calibration in air at 100% relative humidity, to achieve this field probes can be placed in the plastic calibration bottle with a moistened sponge. For the quantification of ammonium excretion we used the phenolhypochlorite method (Rodier 1979). The ammonium production of the shrimps in each experimental condition was calculated as the difference between the final concentration and the initial and is expressed in $\text{mg NH}_4^+ \text{ h}^{-1} \text{ g}^{-1}$ d.w. The 21st chamber was used as a control to measure the oxygen consumption and the ammonium production by the microorganisms and to make the necessary corrections.

The determination of the osmotic pressure was carried out in the shrimps that remained in the reservoirs and they were exposed to the same regimen of salinity fluctuation. Before the salinity changed, 20 shrimps of the reservoir were extracted at random. To each organism a single hemolymph sample of 10 μL was extracted with an automatic pipette directly off the thoraco-abdominal membrane (previously dried with absorbent paper). Osmolality of the hemolymph and the external medium were measured with a Wescor 5520 vapor osmometer, calibration was run a 290 mmol/kg standard and 1000 mmol/kg standard to establish baseline calibration for these standards, and the data were expressed in mmol Kg^{-1} . The linear relationship in the decreased or increased salinity and osmolality at different temperatures were tested using linear regression (Sigma-Stat). The isosmotic points (where hemolymph osmolality is equal to medium osmolality) were determined using the formula of Ferraris et al. (1986). The osmoregulatory capacity (OC), in the blue shrimp was calculated according to Lignot et al. (2000) as the difference between the hemolymph osmolality and the medium osmolality.

Immediately after ending the experiments the shrimps were killed by immersion in boiling water, dried overnight at 60°C , placed on a desiccator for 2 hours and weighed on a balance (OHAUS Explorer) to determine the d.w.

The data of the oxygen consumption and ammonium excretion of the shrimps exposed to the different experimental conditions were plotted in parallel boxes (Tukey 1977). Within the boxes 50% of the data were distributed in the median and the confidence intervals, the other 50% remained distributed in each bar. A 2-way analysis of variance was used as previous determination of the normality and homoscedasticity of the data (Sigma-Stat), to determine the effect of the temperature and salinity fluctuation on the

oxygen consumption, nitrogen excretion and the hemolymph osmotic pressure of *L. stylirostris*.

RESULTS

The oxygen consumption rate in *Litopenaeus stylirostris* juveniles exposed to fluctuations of salinity and maintained in 23°C was 1.1–1.51 mg O₂ h⁻¹ g⁻¹ d.w. This rate was of 1.70–1.98 mg O₂ h⁻¹ g⁻¹ d.w. in organisms acclimated to 28°C and it increased 230% in comparison with those exposed to 33°C (Fig. 1). An analysis of variance indicated that temperature had a significant effect ($P < 0.05$) on the oxygen consumption of the blue shrimp, but the effect of salinity and the interaction temperature-salinity was not significant ($P > 0.05$).

L. stylirostris juveniles exposed to salinity of 40‰ and acclimated to 23°C and 28°C the lower ammonium excretion rate of 0.04–0.08 mg NH₄⁺ h⁻¹ g⁻¹ d.w. was obtained. In the shrimps maintained in 33°C the excretion rate increased significantly up to 0.18 to 0.35 mg NH₄⁺ h⁻¹ g⁻¹ d.w. When the organisms were exposed to descending salinities of 40‰ to 25‰, ammonium excretion increased. In salinity of 16‰ and in the three temperatures in which the shrimps were maintained, the ammonium production increased an average of 220%. When the organisms were exposed to ascending salinities, the excretion of the shrimps was reduced significantly ($P < 0.05$) (Fig. 2). An analysis of variance indicated that there was a significant effect of temperature and salinity on the ammonium excretion rate of *L. stylirostris* ($P < 0.05$), the interaction between temperature and salinity was not significant ($P > 0.05$).

Hemolymph osmolarity of *L. stylirostris* acclimated to 23°C, 28°C, and 33°C and exposure to salinity fluctuation was related linearly; the obtained slopes had an interval of 0.113 to 0.264 (Table 1). When salinity was reduced from 40‰ to 33‰ (1,278–965 mmol Kg⁻¹) the hemolymph concentration was hypo-osmotic with an interval of 873–777 mmol Kg⁻¹ in respect of the external medium. In the interval of salinities of 25‰ to 16‰ (752–556 mmol Kg⁻¹) the hemolymph of the shrimps was hyperosmotic with an interval of 677–770 mmol Kg⁻¹. When the salinity was increased from 25‰ to 40‰ the osmolality of the internal medium of the shrimps was hypo-osmotic with an interval of 737–848 mmol Kg⁻¹ (Fig. 3). An analysis of variance indicated that the fluctuation of salinity had a significant effect ($P < 0.05$) on the hemolymph concentration of the shrimps; the temperature and the interaction between temperature and salinity did not have a significant effect ($P > 0.05$).

The acclimation temperature did not affect significantly ($P > 0.01$) the values of the isosmotic points of the organisms exposed to salinity fluctuation. The isosmotic points obtained in the shrimps exposed to salinity fluctuation had an interval of 726–758 mmol Kg⁻¹ (24.9–25.9‰), when the increased salinity had an interval of 751–766 mmol Kg⁻¹ (25.7–26.2‰) (Fig. 3).

The osmoregulatory capacity (OC) in juveniles of the blue shrimp was modified significantly by salinity ($P < 0.05$) the hemolymph was hypo to hyper-osmotic since in the salinities of (40‰, 32‰, 40‰) had an interval of -417 to -167, whereas the salinities of (25‰, 16‰, 25‰) increased from 18‰ to 143‰ (Fig. 4). The temperature did not affect significantly ($P > 0.05$) the osmoregulatory capacity of the organisms.

DISCUSSION

The respiratory rate in the crustaceans is modified by external factors as salinity, light intensity, dissolved oxygen and tempera-

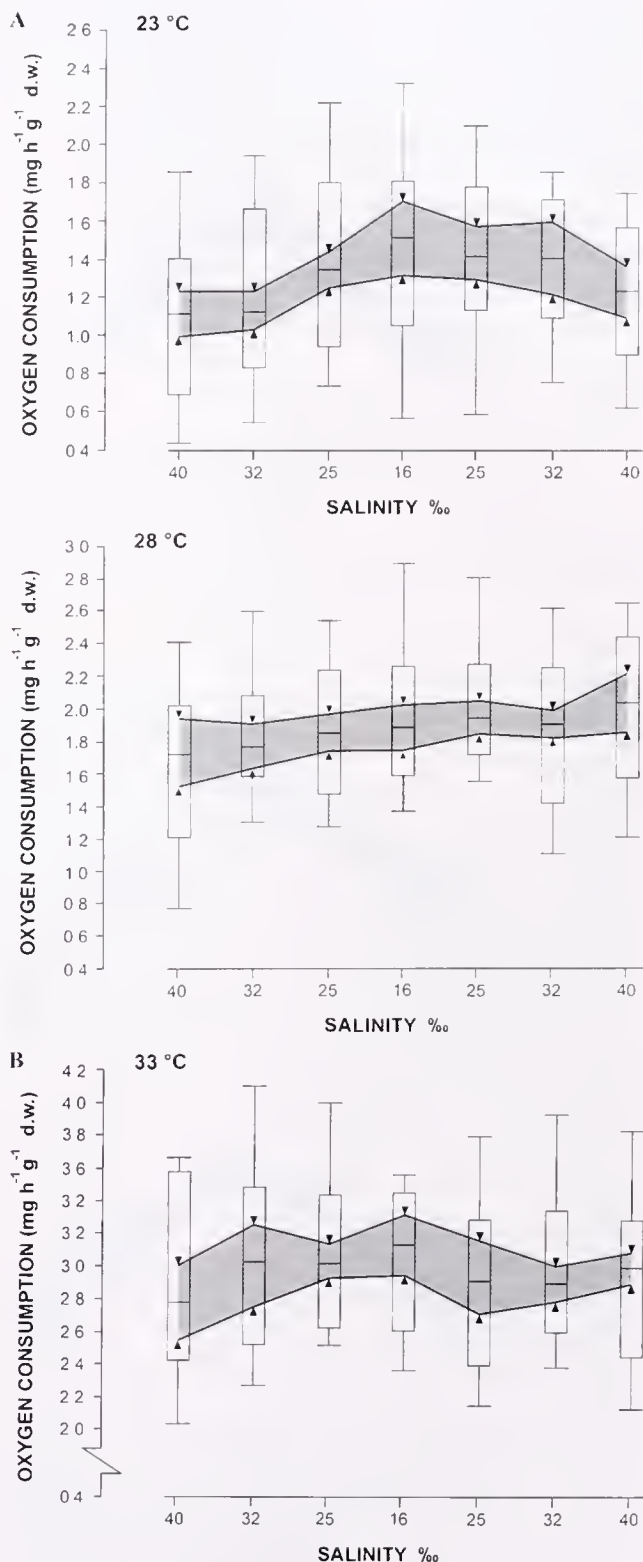


Figure 1. Oxygen consumption rate (mg O₂ h⁻¹ g⁻¹ d.w.) of *Litopenaeus stylirostris* acclimated at temperatures of 23°C, 28°C, and 33°C and exposed to salinity fluctuation. The shaded zone limited by triangles represents the interval of the median. The clear bars involve 50% of the organism distribution and the lines represent the cuartils.

TABLE 1.

Relationship between the hemolymph osmolality (y) and medium osmolality (x) for *Litopenaeus stylirostris* acclimated different temperatures and fluctuating salinities.

Temperature (°C)	Relationship			
	Salinity Decreased	R ²	Salinity Increased	R ²
23	$y = 536.5 + 0.261x$	0.98	$y = 552.6 + 0.264x$	0.89
28	$y = 624.1 + 0.173x$	0.86	$y = 603.1 + 0.197x$	0.90
33	$y = 672.3 + 0.113x$	0.79	$y = 621.7 + 0.189x$	0.96

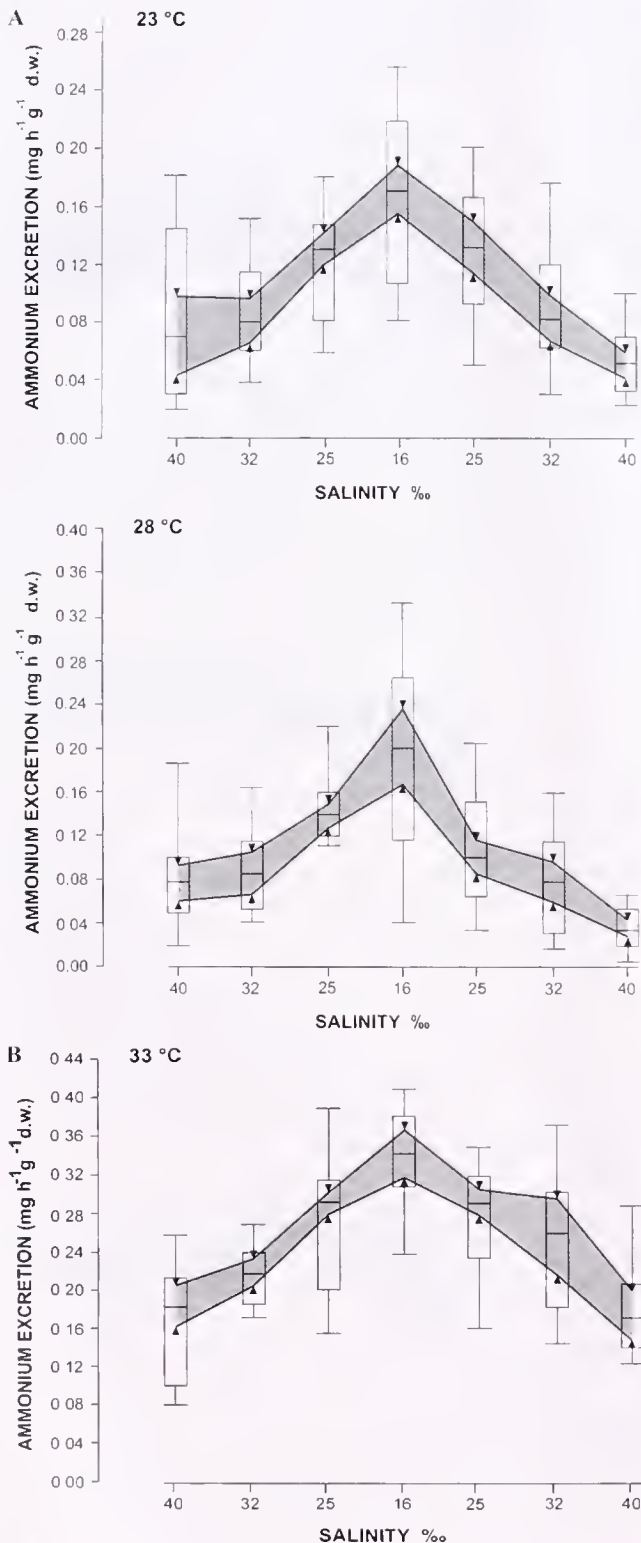


Figure 2. Ammonium excretion rate ($\text{mg NH}_4^+ \text{h}^{-1} \text{g}^{-1} \text{d.w.}$) of *Litopenaeus stylirostris* exposed to combination of temperature and salinity fluctuation. The shaded zone limited by triangles represents the interval of the median. The clear bars involve 50% of the organism distribution and the lines represent the quartils.

ture (Kutty et al. 1971, Bishop et al. 1980, Dalla-Via 1986, Karmaly et al. 1989, Martínez-Palacios et al. 1996). In *Litopenaeus stylirostris* juveniles a direct relationship was obtained between the oxygen consumption rate and temperature. This same response was obtained by Kutty et al. (1971) in *Fenneropenaeus indicus*, in *Penaeus monodon* by Karmaly et al. (1989) and Chen and Lai (1993), in *Farfantepenaeus californiensis* (Holmes) by Villarreal and Ocampo (1993), and Martínez-Palacios et al. (1996) in *Litopenaeus vannamei*. It has been emphasized by Venkataramiah et al. (1974) that the respiratory rate cannot be considered as a good index of the salinity tolerance, except in the cases where the experimental temperature correspond to the natural characteristics of the habitat of the organisms. For blue shrimp a temperature of 28°C corroborates the above-mentioned fact because it corresponds to the preferred temperature determined for this species by Re et al. (2000). Furthermore it is within the average interval experienced in their natural environment.

The oxygen consumption of *L. stylirostris* exposed to salinity fluctuation was not affected significantly. In other euryhaline decapods salinity was not found to significantly affect the oxygen consumption if the experimental organisms were acclimated to the test salinities and if this were not extreme (Bishop et al. 1980, Gaudy & Sloane 1981, Díaz-Herrera et al. 1992, Villarreal & Rivera 1993, Villarreal et al. 1994, Salvato et al. 2001). In aquatic organisms that have been acclimated to a new salinity, Kinne (1967) described four types of metabolic response. The blue shrimp exposed to salinity fluctuation exhibited the type I response because the oxygen consumption was not modified significantly. According to Newell (1976) physiologic rate independence from salinity obtained for blue shrimp is a characteristic of animals that experience highly variable environmental conditions. For other osmoregulator decapods it has been demonstrated, that salinity did not have a pronounced effect on the oxygen consumption if the experimental organisms were acclimated to salinities and if these are not extreme (Bishop et al. 1980, Gaudy & Sloan 1981, Villarreal & Rivera 1993, Salvato et al. 2001). Acclimation time for the *Litopenaeus stylirostris* juveniles to each condition of salinity was sufficient to stabilize their internal medium (Díaz et al. 2001). Furthermore, the intervals of the used salinities correspond to the natural variations to which this species is exposed (Aragón 2000).

In the juveniles exposed to salinity fluctuation ammonium excretion rate was increased, when salinity was reduced from 40‰ to 16‰. This response is related to an increase in the catabolism of the amino acids, because; according to Chen (1998) the shrimps in the lower salinities use proteins as the primary source for requirements of energy. This response has been reported in penaeids such as *M. japonicus*, *F. chinensis*, *P. monodon*, *F. aztecus*, and *L. vannamei* (Spaargaren et al. 1982, Chen & Lin 1992, Chen & Lai

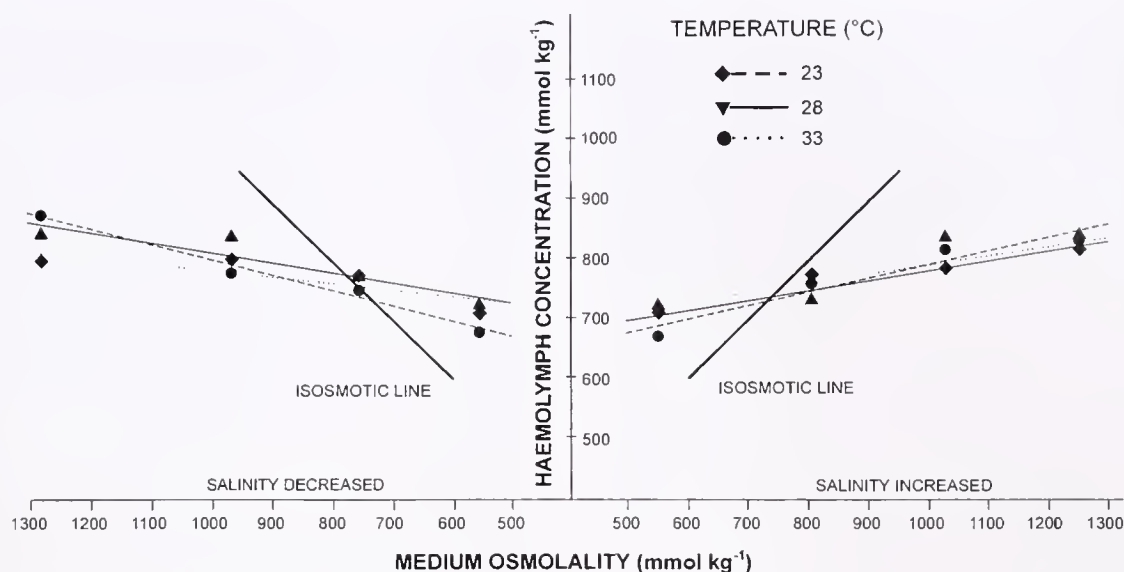


Figure 3. Relation between hemolymph osmolarity of *Litopenaeus stylirostris* and medium osmolarity when they were exposed to combinations of temperature and fluctuating salinities.

1993, Chen et al. 1994, Hernández & Díaz 1995, Jiang et al. 2000, Díaz et al. 2001).

In the blue shrimp exposed to salinity fluctuations, the ammonium excretion rate was related to the processes of osmoregulation, because excretion increased when the shrimps were hyper-regulators and reduced when they were hypo-regulators. It has been demonstrated that there is an increase in the active incorporation of Na^+ followed by the transfer from the organisms to dilute mediums, and this was related to an increase in ammonium excre-

tion because the sodium absorption is required to compensate its lost, caused by the operation of the exchange pump $\text{Na}^+/\text{NH}_4^+$ (Mangum et al. 1976, Pressley et al. 1981). To balance the osmotic concentration of the hemolymph when exposed to dilute mediums, the shrimps use these mechanisms. The quantification of nitrogen excretion rate in the shrimps exposed to the effect of different environmental factors is an important requirement for the design and operation of the intensive production systems (Jiang et al. 2000).

In the *L. stylirostris* juveniles, the osmoregulation pattern obtained was typical of many crustaceans that inhabit the lagoon-estuarine systems. They were hypoosmoregulators and hyperosmoregulators above and below the isosmotic point (Vernberg & Vernberg 1972, Mantel & Farmer 1983). The isosmotic points for the juveniles of the blue shrimp acclimated to different temperatures and exposed to salinity fluctuations had an interval of 726–766 mmol Kg^{-1} (24.9‰ to 26.2‰). These values are within the interval of isosmotic points reported by Brito et al. (2000) and Díaz et al. (2001) in 8 and 11 different species of penaeid respectively. Lemaire et al. (2002) reported an isosmotic point 735 mmol Kg^{-1} for juvenile shrimp *L. stylirostris* maintained in constant salinities. In the *L. vannamei* juveniles exposed to salinity fluctuations and acclimated to different temperatures, Díaz et al. (2001) obtained that the isosmotic points had an interval of 712–777 mmol Kg^{-1} . These values are greater than those reported by Castille and Lawrence (1981) and Rodríguez (1981) of 680 (24‰) and 610 (21‰) mmol Kg^{-1} respectively. The differences can be attributed to the different experimental conditions used in these studies, because it is known that factors like temperature, dissolved oxygen, molt stage, the size of the organisms, and the nutritional state have an influence on the hemolymph osmolality control of the penaeid shrimp (Williams 1960, Charmantier et al. 1994, Lignot et al. 1999). Our observations were conducted under standardized experimental conditions, and allow us to isolate the effect of temperature salinity fluctuations and repeated the experiments.

To evaluate the osmoregulatory capacity of *L. stylirostris* the values of the slopes of the relationship between the osmotic concentration of the medium and the hemolymph were compared with

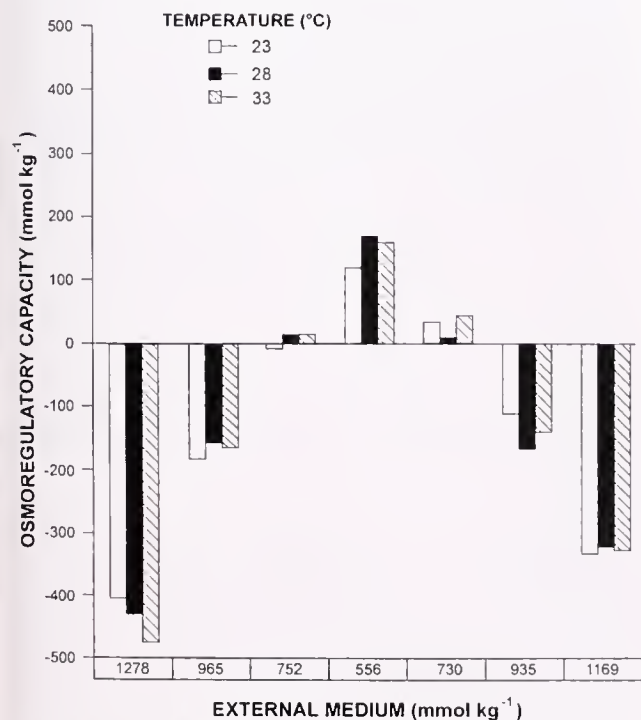


Figure 4. Osmoregulatory capacity of blue shrimp *Litopenaeus stylirostris* acclimated at 23, 28 y 33°C and exposed to fluctuating salinities.

those obtained for other penaeids maintained in constant salinities, which had an interval of 0.11–0.73 (Díaz et al. 2001). A deviation of the slope of the isosmotic line reflects the degree of the regulation capacity (slope = 0 osmoregulator; slope = 1 osmoconformer). The values of the slopes of the juveniles acclimated to 23°C, 28°C, and 33°C and exposed to decreasing salinity were 0.26‰, 0.17‰, and 0.11‰ and for ascending salinities 0.26‰, 0.19‰, and 0.18‰, respectively. The organisms with higher slopes as those maintained in 23°C had a weak regulation and their osmoregulatory capacity was poor. According to Vernberg and Silverthorn, (1979) the temperature affect osmoregulatory capacity in crustacean by influencing water movement across cell membrane and in the uptake and loss of ions. The lower values of the slopes for the organisms maintained in 28°C and 33°C reflected that in these conditions the shrimps had a hyperosmoregulatory capacity in higher salinities and hyperosmoregulatory in lower salinity. Therefore, they can be characterized as strong regulators, because they adapt rapidly to the new salinities by increasing and decreasing the hemolymph osmotic concentration. For *L. stylirostris* subadults, Lemaire et al. (2002) obtained that reducing the temperature from 26°C to 15°C, there was a significant decrease of the osmoregulatory capacity in organisms maintained in con-

stant salinities. This different response can be considered from the adaptive point of view because the lower sensibility in the osmoregulatory capacity shown by the juveniles permits living in the lagoon-estuarine environment in which there are significant variations of temperature.

To optimize the culture of the blue shrimp in controlled conditions it is recommended that temperatures of approximately 28°C be used. Because the best salinity has not been determined experimentally, we propose that 25‰ be considered as the optima for this species, because it corresponds with the isosmotic point determined for the blue shrimp. According to Pannikar (1968), in this condition the shrimps expend less energy for the maintenance of the osmotic gradient and therefore the growth increases when the organisms are cultivated in a stress-free environment.

ACKNOWLEDGMENTS

The authors thank the Laboratory El Camarón Dorado located in Huatabampo, Sonora, for the donation of the specimens used in this investigation. SIMAC-CONACYT gave the economical support to the projects 98011110606 and 200017509. We also appreciate the graphical material; elaborated by Jose M. Domínguez and Francisco J. Ponce from the Drawing Department of CICESE.

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MOLECULAR PHYLOGENY OF SUPERFAMILY PENAEOIDEA RAFINESQUE-SCHMALTZ, 1815, BASED ON MITOCHONDRIAL 16S PARTIAL SEQUENCE ANALYSIS

ANA R. VÁZQUEZ-BADER,¹ JULIO C. CARRERO,² MARTÍN GARCÍA-VARELA,²
ADOLFO GRACIA^{1*} AND JUAN P. LACLETTE²

¹Dept. of Marine Ecology, Instituto de Ciencias del Mar y Limnología UNAM, A. P. 70305, 04510 México, D.F. México; ²Dept. of Immunology, Instituto de Investigaciones Biomédicas, UNAM, 04510 México D.F., México.

ABSTRACT Partial mitochondrial DNA sequences were analyzed to reconstruct the phylogeny of Superfamily Penaeoidea. In addition to 11 new 16S rRNA sequences generated for this study, 18 previously reported sequences (including 3 outgroups) were examined. The phylogenetic relationships estimated through maximum likelihood and neighbor joining methods supported the monophyly of the Superfamily Penaeoidea. Other findings, challenge our current classification schemes of the superfamily, and are presented as hypothesis for future work: the paraphyly of Penaeidae family and its close relationship to Solenoceridae; the close association between Aristeidae, Benthescymidae and Sicyoniidae questions their erection as separated families.

KEY WORDS: molecular phylogeny, 16S, mitochondrial DNA, Dendrobranchiata, Penaeoidea, shrimp

INTRODUCTION

Penaeoids shrimp of the Superfamily Penaeoidea Rafinesque-Schmaltz, 1815, are a diverse and abundant group with more than 400 species currently recognized within 49 genera representing 5 families: Aristeidae, Wood-Mason 1891; Benthescymidae, Wood-Mason 1891; Penaeidae, Rafinesque-Schmaltz 1815; Sicyoniidae, Ortmann 1898; and Solenoceridae, Wood-Mason 1891. Members of Penaeidae and Sicyoniidae are predominantly found in littoral water and include most of the shrimp commercially caught around the world in tropical and subtropical areas, whereas Aristeidae, Benthescymidae, and Solenoceridae are either deep benthic dwellers or members of the meso- and bathypelagic-fauna. Penaeoids shrimp show a diverse and complex morphology (i.e., the male and female genitalia), which have been recently reviewed by Pérez-Farfante and Kensley (1997).

The presence of a nauplius larval stage as well as the fossil record that placed penaeoids as the most basal group within decapods originated in the Permian-Triassic (Felgenhauer & Abele 1983) or in the Carboniferous (Schram 1977, Schram 1982, Dall et al. 1990). One hypothesis on the evolutionary relationships within Penaeoidea has been advanced (Burkenroad 1983), posing an Aeger-like organism as the common ancestor of all penaeoids, from which 2 branches arose: one early diverging branch leading to penaeids and sicyoniids, and a later emerging group resulting in the modern solenocerids, aristeids, and benthescymids.

In spite of their commercial, ecological, evolutionary, and taxonomical significance, no attempts have been made to elucidate the phylogeny of Superfamily Penaeoidea. A number of molecular studies have been carried out on the phylogeny and population genetics of Penaeidae (e.g., Bauer 1986, Bauer 1991, Palumbi & Benzie 1991, Machado et al. 1993, Bouchon et al. 1994, García et al. 1996, Baldwin et al. 1998, Ball et al. 1998, Tassanakajon et al. 1998, Tong et al. 2000, Chu et al. 2003). Other four families have not been approached through molecular phylogenetics.

This study is the first attempt to contribute a preliminary molecular phylogeny for the Superfamily Penaeoidea through analysis of partial sequences of 16S rRNA mitochondrial genes.

MATERIAL AND METHODS

Collection of Specimens

Eleven species representing the 5 families of Superfamily Penaeoidea that were included in the study: *Aristeus antillensis*, Milne Edwards and Bouvier 1909; *Aristaeopsis edwardsiana*, (Johnson 1867); and *Penaeopsis serrata*, Bate 1881 (collected during the oceanographic cruise BATO, Biota de los Arrecifes de la Plataforma y Talud continental en el noroeste del Banco de Campeche, Golfo de México); *Sicyonia burkenroadi*, Cobb 1971; *Sicyonia dorsalis*, Kingsley 1878; *Sicyonia brevirostris*, Stimpson 1871; *Farfantepenaeus duorarum* (Burkenroad 1939); *Litopenaeus setiferus* (Linnaeus 1767); and *Solenocera vioscai*, Burkenroad 1934 were caught during the oceanographic cruise SGM-6 (Banco y Sonda de Campeche, Golfo de México), both cruises were carried on board the R/V *Justo Sierra* (Instituto de Ciencias del Mar y Limnología, UNAM). *Gemadas* sp., and *Bentheogen-nema intermedia* (Bate 1888), were collected during the oceanographic cruise DgoMB (Deep Gulf of Mexico Benthos Study), carried on board the R/V *GYRE* (Texas A & M, University), and identified by M. Wickstein. All samples consisted of a piece of pleopod (70–80 mg) preserved either in 70% ethanol or in liquid nitrogen.

DNA Isolation and Amplification

DNA extractions were carried out using the Blood & Cell Culture DNA Mini Kit (Qiagen), following the instruction from the manufacturer. A 418-bp fragment of the 16S gene was amplified through polymerase chain reaction performed in a BioRad Mastercycler Gradient. The 50- μ l reaction mixture contained: 0.2 mM each dNTP (Gibco BRL), \times 1 Amplification Buffer (Invitrogen), 1 mM MgSO₄ (Invitrogen), 1 μ M each primer (Invitrogen), 0.5 unit Platinum Pfx DNA Polymerase (Invitrogen), and 80–100 ng template DNA (undiluted or diluted 10–20 \times in ddH₂O). Two primers were developed for this study and used to amplify the homologous 3' end of 16S mitochondrial region: Forward E 5'-TAGAGAATTCGACCGTGCGAAGGTAGC-3'; Reverse X 5'-TTGAGAGCTCATTC AACATCGAGGTGGC-3'. These primers contained EcoRI and XhoI sites, respectively, to facilitate ligation into standard plasmids for sequencing, although, in this report all sequencing was carried out from PCR products. Cycling condi-

*Corresponding author. E-mail: gracia@mar.icmyl.unam.mx

tions were: 94°C/4 min initial denaturing step followed by 30 cycles of 94°C/1 min, 45 to 55°C/1 min, 72°C/1 min, and final extension step of 72°C/10 min. Doubled-stranded PCR products were purified from gel (Montage DNA Gel Extraction Kit, Millipore) or directly from the PCR reaction mixture (Microcon-PCR Filter Unit, Millipore).

Purified PCR products (10 µl) were sequenced in both directions using the ABI- Big Dye-Terminator Sequencing Kit (Applied Biosystem Inc.) under manufacturer-recommended reaction conditions.

Sequence Analysis

All nucleotide sequences were aligned using Clustal W 1.5 c (Thompson et al. 1994) and then manually adjusted. Phylogenetic analysis was initially carried out with PAUP* 4.10 (Swofford 2003). To determine which model of sequence evolution best fitted the data set, a nested likelihood ratio test was performed using Modeltest program version 3.0 (Posada & Crandall 1998). After the evolution model was determined, phylogenetic relationships were inferred using maximum likelihood (Felsenstein 1981). Fifty random taxon addition heuristic searches with tree bisection-reconnection branch swapping were conducted. To support the relationships among the taxa 100 bootstrap searches were performed (Felsenstein 1985). Figure was prepared in part using the programs RETREE and DRAWGRAM from PHYLIP (Felsenstein 1999). Phylogenetic relationships were also estimated using neighbor-joining (NJ) analysis (Saitou & Nei 1987). NJ analysis was performed using Jukes-Cantor and pair-wise deletion options (MEGA 2.0, Kumar et al. 1993). Bootstrapping (1000 replicates) was performed to assess the confidence level at each branch.

RESULTS

In addition to 11 new mitochondrial 16S partial sequences generated for this study, another 15 partial sequences from 5 different Penaeoidea families, and 3 outgroup taxa were also included. Our dataset consisted of 29 sequences, 303–374 bp long. New sequences have been deposited in GenBank under accession numbers shown in Table 1.

Aligned sequences were considerably AT-rich (75%). Base composition was A = 0.37, C = 0.026, G = 0.17, and T = 0.38. The average distance in all Penaeoidea species was 0.137 ± 0.12 and ranged from 0.001 between *Sicyonia dorsalis* and *Aristaeopsis edwardsiana* to 0.234 between *Aristeus antillensis* and *Farfantepenaeus duorarum* as well as *A. antillensis* and *Litopenaeus setiferus* (Table 2).

The likelihood ratio test indicated that the model with best fit for the data set was the Hasegawa-Kishino-Yano model (HKY; Hasegawa et al. 1985), with a heterogeneity rate: (+G; Yang 1994); a transition/transversion ratio of 2.1355; a gamma shape parameter of 0.44; and with invariable sites (+I) of 0.2147. Maximum likelihood analysis using this model yielded two trees with a -ln likelihood of 2942.592. Differences among both trees were the position of *Aristeus antillensis*, *Aristaeopsis edwardsiana*, and *S. dorsalis*. Despite the number of polytomies in the ML tree (Fig. 1), several features can be observed, being the monophyly of the Penaeoidea superfamily the most conspicuous, well apart from the 3 outgroup species: *Synalpheus pectiniger*, *Cataleptodius floridanus*, and *Xantho poressa*. Surprisingly, the Penaeidae family appeared as a paraphyletic assemblage formed by at least 3 clades. Clade A, formed by 9 species within 6 genera (according to Pérez-Farfante & Kensley 1997) including the three species of American

TABLE 1.

List of the Superfamily Penaeoidea species used in this study.

Family/Species	Zoogeographic Distribution	GenBank Accession Numbers
Aristeidae		
<i>Aristaeopsis edwardsiana</i> * (<i>A. edw</i>)	WA, EA, IP	AY601734
<i>Aristeus antillensis</i> * (<i>A. ant</i>)	WA	AY601738
Benthescymidae		
<i>Bentheogennema intermedia</i> * (<i>B. int</i>)	WA, EA, EP, IP	AY601737
<i>Gennadas</i> sp* (<i>Genn</i>)	WA, EP, IP, EA	AY601739
Penaeidae		
<i>Farfantepenaeus aztecus</i> (<i>F. azt</i>)	WA	AF279811
<i>Farfantepenaeus brasiliensis</i> (<i>F. bra</i>)	WA	AF192053
<i>Farfantepenaeus duorarum</i> * (<i>F. duo</i>)	WA	AY601732
<i>Fenneropenaeus merguensis</i> (<i>F. mer</i>)	EA, IP	AF279814
<i>Litopenaeus setiferus</i> * (<i>L. set</i>)	WA	AY601735
<i>Litopenaeus vannamei</i> (<i>L. van</i>)	EP	AF279818
<i>Marsupenaeus japonicus</i> (<i>M. jap</i>)	IP	AF279820
<i>Melicertus canaliculatus</i> (<i>M. can</i>)	IP	AF279825
<i>Metapenaeopsis barbata</i> (<i>M. bar</i>)	IP	AY264905
<i>Metapenaeus ensis</i> (<i>M. ens</i>)	IP	AF279810
<i>Parapenaeopsis hardwickii</i> (<i>P. har</i>)	IP	AY264910
<i>Parapenaeus fissurus</i> (<i>P. fis</i>)	IP	AY264909
<i>Penaeopsis serrata</i> * (<i>P. ser</i>)	WA, EA	AY601733
<i>Penaeus monodon</i> (<i>P. mon</i>)	IP	AF279829
<i>Trachysalambria curvirostris</i> (<i>T. cur</i>)	EA, IP	AY264916
<i>Xiphopenaeus kroeyeri</i> (<i>X. kro</i>)	EP, WA	AF192092
Solenoceridae		
<i>Solenocera crassicornis</i> (<i>S. cra</i>)	IP	AY264915
<i>Solenocera koelbeli</i> (<i>S. koel</i>)	IP	AF105038
<i>Solenocera vioscai</i> * (<i>S. via</i>)	WA	AY601736
Sicyoniidae		
<i>Sicyonia brevirostris</i> * (<i>S. bre</i>)	WA, EP	AY601742
<i>Sicyonia burkenroadi</i> * (<i>S. bur</i>)	WA	AY601741
<i>Sicyonia dorsalis</i> * (<i>S. bur</i>)	WA	AY601740
Outgroups		
<i>Synalpheus pectiniger</i> (<i>S. pec</i>)	WA	AF230259
<i>Cataleptodius floridanus</i> (<i>C. flo</i>)	WA	AJ274698
<i>Xantho poressa</i> (<i>X. por</i>)	WA	AJ130814

Abbreviation for the species names are shown in brackets.

* Species sequenced for this work.

WA, Western Atlantic; EA, Eastern Atlantic; EP, Eastern Pacific; IP, Indo-West Pacific.

Farfantepenaeus and the two *Litopenaeus* species, the Indo-West Pacific *Melicertus canaliculatus* and *Marsupenaeus japonicus*, as well as 2 separated species, *Penaeus monodon* and *Fenneropenaeus indicus*, which were not resolved in the ML tree, although clearly separated from the other genera. Clade B consisted of 2 Indo-West Pacific species *Trachysalambria curvirostris* and *Parapenaeopsis hardwickii*, and the western Atlantic *Xiphopenaeus kroeyeri*. Clade C included *Parapenaeus fissurus* and *Metapenaeopsis barbata* from the Indo-West Pacific and *Penaeopsis serrata* from the western Atlantic, unexpectedly associated with the species of the Solenoceridae family: *Solenocera koelbeli* and *S. crassicornis* from the Indo-West Pacific, and *S. vioscai* from the Western Atlantic. The only species of the Penaeidae family that appeared isolated was *Metapenaeus ensis*, which showed the highest genetic distances within Penaeidae (see Table 2). Another remarkable feature from the ML tree was the grouping of species belonging to families Sicyoniidae, Benthescymidae, and Aristeidae in Clade D. Finally, the three outgroup species appeared

TABLE 2.

Pairwise mitochondrial 16S sequence divergence for 26 Penaeoidea species and three outgroups. Data were calculated using Jukes-Cantor distance parameter (MEGA2.0). Abbreviations for species are shown in Table 1.

	<i>C. flo</i>	<i>X. por</i>	<i>S. pec</i>	<i>F. bra</i>	<i>L. van</i>	<i>F. act</i>	<i>T. cur</i>	<i>P. har</i>	<i>P. fis</i>	<i>M. ens</i>	<i>M. can</i>	<i>M. jap</i>	<i>F. mer</i>	<i>X. kro</i>	<i>P. mon</i>	<i>F. duo</i>	<i>P. ser</i>	<i>L. set</i>	<i>M. har</i>	<i>S. vio</i>	<i>S. koe</i>	<i>S. cra</i>	<i>A. ant</i>	<i>A. edw</i>	<i>Gen</i>	<i>B. int</i>	<i>S. dor</i>	<i>S. har</i>	<i>S. bre</i>	
<i>C. flo</i>	—																													
<i>X. por</i>	0.129	—																												
<i>S. pec</i>	0.290	0.307	—																											
<i>F. bra</i>	0.287	0.314	0.290	—																										
<i>L. van</i>	0.281	0.298	0.288	0.100	—																									
<i>F. act</i>	0.295	0.328	0.315	0.047	0.088	—																								
<i>T. cur</i>	0.253	0.302	0.264	0.129	0.122	0.119	—																							
<i>P. har</i>	0.266	0.333	0.237	0.173	0.162	0.172	0.079	—																						
<i>P. fis</i>	0.253	0.306	0.280	0.154	0.109	0.135	0.067	0.107	—																					
<i>M. ens</i>	0.283	0.305	0.286	0.120	0.159	0.166	0.097	0.120	0.115	—																				
<i>M. can</i>	0.268	0.304	0.336	0.127	0.126	0.126	0.119	0.148	0.126	0.149	—																			
<i>M. jap</i>	0.277	0.313	0.336	0.094	0.136	0.133	0.119	0.155	0.123	0.152	0.017	—																		
<i>F. mer</i>	0.267	0.311	0.282	0.149	0.097	0.103	0.124	0.142	0.109	0.146	0.011	0.109	—																	
<i>X. kro</i>	0.273	0.310	0.276	0.103	0.144	0.141	0.047	0.094	0.094	0.126	0.136	0.143	0.141	—																
<i>P. mon</i>	0.280	0.309	0.272	0.067	0.119	0.124	0.102	0.126	0.106	0.136	0.118	0.112	0.078	0.134	—															
<i>F. duo</i>	0.307	0.332	0.285	0.126	0.103	0.055	0.118	0.175	0.128	0.166	0.136	0.132	0.121	0.138	0.124	—														
<i>P. ser</i>	0.241	0.297	0.276	0.106	0.097	0.119	0.082	0.119	0.047	0.132	0.116	0.126	0.100	0.100	0.112	0.128	—													
<i>L. set</i>	0.278	0.306	0.308	0.142	0.070	0.111	0.150	0.182	0.125	0.166	0.157	0.164	0.105	0.164	0.127	0.123	0.115	—												
<i>M. har</i>	0.261	0.309	0.288	0.134	0.125	0.132	0.100	0.138	0.058	0.141	0.143	0.154	0.125	0.118	0.128	0.154	0.061	0.141	—											
<i>S. vio</i>	0.273	0.302	0.309	0.147	0.133	0.140	0.113	0.143	0.101	0.127	0.124	0.127	0.136	0.123	0.123	0.129	0.073	0.156	0.104	—										
<i>S. koe</i>	0.278	0.310	0.307	0.149	0.139	0.152	0.122	0.148	0.082	0.138	0.148	0.144	0.129	0.135	0.126	0.155	0.093	0.148	0.100	0.062	—									
<i>S. cra</i>	0.281	0.305	0.306	0.210	0.138	0.155	0.125	0.148	0.081	0.141	0.151	0.147	0.128	0.134	0.125	0.158	0.096	0.148	0.099	0.065	0.003	—								
<i>A. ant</i>	0.337	0.370	0.371	0.196	0.222	0.226	0.142	0.197	0.159	0.169	0.207	0.198	0.186	0.175	0.156	0.234	0.163	0.234	0.176	0.176	0.172	0.176	—							
<i>A. edw</i>	0.325	0.357	0.368	0.184	0.212	0.216	0.132	0.188	0.152	0.153	0.193	0.185	0.173	0.164	0.145	0.224	0.156	0.228	0.169	0.169	0.165	0.168	0.007	—						
<i>Gen</i>	0.307	0.335	0.340	0.184	0.198	0.201	0.132	0.178	0.147	0.151	0.197	0.189	0.165	0.163	0.140	0.208	0.150	0.212	0.161	0.161	0.161	0.164	0.017	0.010	—					
<i>B. int</i>	0.306	0.326	0.339	0.183	0.198	0.194	0.129	0.175	0.143	0.144	0.189	0.181	0.161	0.156	0.136	0.201	0.147	0.208	0.157	0.157	0.157	0.160	0.007	0.003	0.011	0.006	—			
<i>S. dor</i>	0.298	0.326	0.335	0.181	0.195	0.198	0.129	0.175	0.143	0.144	0.189	0.181	0.161	0.160	0.136	0.204	0.147	0.208	0.157	0.157	0.157	0.160	0.007	0.000	0.011	0.006	—			
<i>S. har</i>	0.303	0.330	0.335	0.184	0.198	0.201	0.132	0.178	0.147	0.148	0.193	0.185	0.165	0.163	0.140	0.208	0.150	0.212	0.161	0.161	0.161	0.164	0.010	0.003	0.014	0.008	0.008	—		
<i>S. bre</i>	0.313	0.341	0.335	0.192	0.187	0.205	0.150	0.193	0.157	0.155	0.201	0.193	0.157	0.171	0.147	0.208	0.154	0.216	0.162	0.162	0.165	0.165	0.077	0.067	0.068	0.062	0.062	0.065	—	

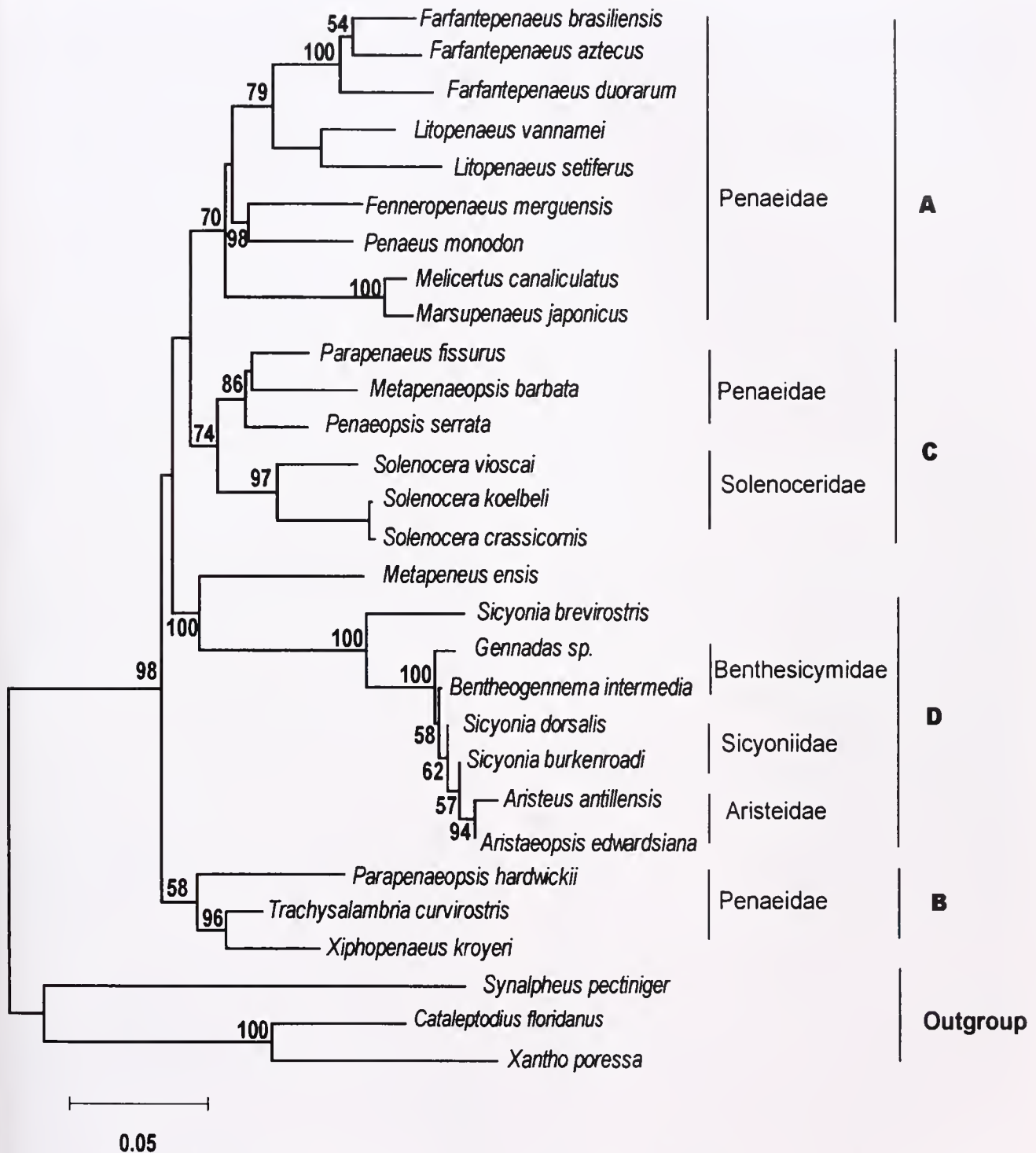


Figure 2. Neighbor-joining (NJ) tree from 16S rRNA data. Bootstrap values are shown as percentages and are based on 1,000 replicates. Branches without bootstrap numbers mean that the bootstrap values are below 50%. Labels A, B, C, and D indicate the major clades of the Superfamily Penaeoidea.

emerged from both analyses and might be erected as hypothesis for future work using larger sequence data: (1) The monophyly of Superfamily Penaeoidea; (2) The paraphyly of Penaeidae family and its close relationship to Solenoceridae family; and (3) Aristeidae, Benthescymidae and Sicyoniidae form a compact group with small genetic distances and might not deserve erection as separated families.

The relatively large genetic divergence observed within family Penaeidae (Clades A-C) seems to be in agreement with morphologic traits described long ago (Burkenroad 1936, Burkenroad 1983, Kubbo 1949). Members of clade A showed the greatest mean divergence (0.0108); taken as a natural group, they might be characterized morphologically by the presence of an epipod on third maxilliped, pleurobranchiae on fifth pereopod, ventral and

dorsal rostral teeth, as well as by the absence of branchiostegal, pterygostomian and parapenaeid spines, and transverse and longitudinal sutures on carapace. All but the two species of *Litopenaeus* within clade A, show a symmetrical and semiclosed petasma without distolateral projections, uninvginated and unpaired seminal receptacles, simple spermatophore without accessories, and thelycum closed with two lateral plates (with the exception of *Marsupenaeus*, which shows a thelycum with one plate, and spermatophore with accessories). The *Litopenaeus* species have a semiopen petasma and thelycum without seminal receptacles, and a complex spermatophore with accessories. Our results are in agreement with a previous study based on 16S mitochondrial DNA (Maggioni et al. 2001), proposing similar differentiation between *Farfantepenaeus* with closed thelycum and semiclosed petasma versus *Litopenaeus* with open thelycum and semiopen petasma.

Clade B formed by *Trachysalambria*, *Parapenaeopsis*, and *Xiphopenaeus* appeared divergent from the other penaeids. They are morphologically different from Clade A by the absence of pleurobranchiae on pereopods 4 and 5, the lack of epipod on third maxilliped, presence of rostral teeth only on dorsal side, symmetrical and semiclosed petasma with hornlike or spoutlike distolateral projections, closed thelycum with one lateral plate, and the seminal receptacles paired and invaginated.

Clade C differs from Clades A and B by the lack of pleurobranchia only on fifth pereopod and the presence of pterygostomian and parapenaeid spines. However, members of clade C are morphologically similar to those in clade B in several sexual characters: symmetrical and semiclosed petasma with invaginated and paired seminal receptacles, closed thelycum with one plate and simple spermatophore without accessories (except for *Metapenaeopsis* which shows asymmetric petasma and uninvginated and unpaired seminal receptacles).

Association of family Solenoceridae deserves further attention because the uniqueness of their postorbital spine within Superfamily Penaeoidea. Relationships between solenocerids and penaeoids suggested in our analyses were surprising, although are not without precedent. Burkenroad (1983) proposed that some genera within Solenoceridae (e.g., *Haliporus*), are closer to Aristeidae (by the presence of appendix interna, the two arthrobranchiae on first maxilliped and the mesial tubercle on optic calathus), whereas others (e.g., *Solenocera* and *Pleoticus*) resemble Penaeidae by the presence of pterygostomian, brachiostegal and ocular spines, an orbital scale, similar branchial formula, a semiopen petasma, and an open thelycum. Our results certainly differ from previous systematic studies and might suggest that morphologic characters used to differentiate among families within Superfamily Penaeoidea, have been over-emphasized. Further molecular analyses with larger sequence data and including other genera of Solenoceridae, will be necessary to elucidate the status of this family.

The apparently anomalous position of *M. ensis* within penaeids seems to agree with some atypical morphologic characters like the petasma with distolateral projections, the lack of pleurobranchia and exopod on fifth pereopod, the fifth male pereopod modified and the presence of basial spine on third maxilliped and pereopods 2–3. On this respect, previous study using allozyme markers (Tam & Chu 1993) have also revealed considerable genetic differences between *Metapenaeus* and other subgenera of *Penaeus*.

The largest genetic distance (mean = 0.175), was found between the compact group (Clade D) formed by Sicyoniidae, Benthescymidae, and Aristeidae and the other penaeoid families. Our results strongly suggest that the 3 families are closely related and separated from families Penaeidae and Solenoceridae, however, the status of each family within Clade D will require further molecular analysis. Aristeidae and Benthescymidae share a similar branchial formula and presence of a reduced prosartema, but differ in features of the genitalia: *A. antillensis* and *A. edwardsiana* show open thelycum and petasma, whereas *B. intermedia* and *Gemadas* sp. have closed petasma and thelycum with invaginated and paired seminal receptacles. Nevertheless, the seminal receptacles and the spermatophores in Benthescymidae are not homologous with Penaeidae and Sicyoniidae (Burkenroad 1936, Bauer 1986, Bauer 1991). Species of *Sicyonia*, resemble those of Penaeidae in some genitalia characteristics, whereas with Aristeidae and Benthescymidae share the lack of exopods in the pereopods 1–5, and absence of the ocular scale. On the other hand, sicyoniids possess morphologic characters that otherwise are unique to Penaeoidea: a single pleurobranchia on the fifth pereopod, uniramous third to fifth pleopods, lack of exopods in all pereopods, closed petasma with distal, sclerotized projections, without true spermatopores, and thelycum with lanceolate median plate.

A recent molecular analysis of the phylogenetic relationships of genus *Penaeus* s.l. based in partial sequences of 16S and Cytochrome oxidase I (COI) is consistent with our results within clade A (Lavery et al. 2004).

As indicated earlier, the value of this study is to direct the attention to three major questions about Superfamily Penaeoidea, and in particular, about the status and relationships among families. Additional molecular studies are needed to provide stronger evidence on the evolutionary history within the superfamily.

ACKNOWLEDGMENTS

The authors thank P. de la Torre, A. Gómez, and C. Padilla for the technical support and field work. Special gratitude is extended to D. Piñero for kindly introducing me to Molecular Biology Research (A. R. V. B.). This work was partially supported by research grants from CONACYT (41693-M: J. P. L. and J. C. C.), DGAPA UNAM (IN206102-3: J.P.L. and J. C. C.) and Fundación Miguel Alemán, A.C. (J. P. L.).

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EFFECTS OF FEEDING LEVEL ON THE GROWTH, ENERGY BUDGET AND BODY BIOCHEMICAL COMPOSITION OF CHINESE SHRIMP *FENNEROPENAEUS CHINENSIS*

GUOQIANG HUANG, SHUANGLIN DONG* AND FANG WANG

Mariculture Research laboratory, Fisheries College, Ocean University of China, Qingdao 266003, People's Republic of China

ABSTRACT Chinese shrimp *Fenneropenaeus chinensis* with wet weight of 3.0 g, 8.0 g, 14.2 g, and 19.0 g were fed at 5 feeding levels of 0, 0.5%, 1.0%, and 1.5% of the body weight, and *ad libitum* to investigate the growth and food conversion efficiency under different feeding levels. The relationship among daily growth coefficient (DGC) (in terms of wet weight, dry weight, protein, and energy), initial body weight (IBW) and feeding level (FL) was well described with the dualistic linear model $DGC = B_0 + B_1 \times IS_w + B_2 \times FL$. The relationship between food efficiency (FE) in terms of dry weight, protein, and energy of shrimp with different body weight and feeding level was described with the model $FC = B_0 + B_1 \times \ln FL$. DGC and IBW were negatively correlated, regardless of term of wet weight, dry weight, protein, or energy of the shrimp body weight. The maintenance feeding level (MFL) of Chinese shrimp was positively linear correlated to IBW and the maintainable energy intakes (MEI, J/g BW/d) for shrimp with different IBW were: 3.0 g, 218.3 J/g BW/d; 8.0 g, 213.5 J/g BW/d; 14.2 g, 207.5 J/g BW/d; 19.0 g, 202.9 J/g BW/d.

KEY WORDS: feeding level, daily growth coefficient, energy budget, body biochemical composition, Chinese shrimp

INTRODUCTION

Either in natural environment or culture ponds, the crustacean experience starvation and under-nutrition occasionally. Studies have found that starvation caused low metabolic rate (Wallace 1973, Regnault 1981, Whyte et al. 1986, Chappelle et al. 1994) and high body moisture content (Dall 1974, Cuzon et al. 1980, Barclay et al. 1983, Sasaki et al. 1986, Stuck et al. 1996a, Wu & Dong 2000, Wu & Dong 2002a, Wu & Dong 2002b, Lin et al. 2004) in crustacean. Other items of shrimp body composition such as protein, lipid, and energy content decrease gradually through the fasting period (Barclay et al. 1983, Stuck et al. 1996a, Wu et al. 2000, Wu & Dong 2002a, Wu & Dong 2002b, Lin et al. 2004). Positive correlation existed between the growth and feeding level in shrimp and crabs (Bartley et al. 1980, Miao & Tu 1993, Gu et al. 1996). Besides, the feeding level affected body composition, such as moisture, protein, and lipid content of American lobster *Homarus americanus* (Bartley et al. 1980) and redclaw crayfish, *Cherax quadricarinatus* (Gu et al. 1996). To explore the physio-ecologic characters of shrimp in different nutritional conditions, the responses of growth, FE, energy budget, body biochemical composition, and body gross energy content to feeding levels of Chinese shrimp, *Fenneropenaeus chinensis* with different body weight, were studied in this experiment.

MATERIALS AND METHODS

Diet Used in This Experiment

The diet used in this study was a commercial formulated shrimp pellet (Sea-Horse Brand; Producer: Fujian Mawei Unite Feed Ltd. Co., China). The proximal composition of the diet was: moisture, 7.70%; crude protein, 42.57%; crude lipid, 9.93%; ash, 10.75%; and gross energy 19.23 KJ.g⁻¹.

Rearing Conditions

Chinese shrimp were kept in glass aquaria (45 × 30 × 30 cm³, water volume of 35 dm³), and each rearing unit was stocked with 1 shrimp. The room temperature was thermostatically controlled,

and water temperature was 25° ± 05°. Aeration was provided continuously and 1/2 to 2/3 of water was exchanged every other day. Seawater used in the experiment was filtered by composite sand filter. During the experiment, dissolved oxygen water was maintained above 5.5 mg/L, pH was about 8.0, the water salinity was between 30‰ to 33‰, and the photoperiod of 14 hours of light: 10 hours of darkness was used.

Source and Acclimation of Shrimp

The experiment was carried out at the Mariculture Research Laboratory, Ocean University of China, People's Republic of China. The shrimp used in the experiment were collected from the Tianheng Shrimp Farm, Qingdao. Prior to the experiment, the shrimp were transferred into aquaria and underwent a 7-day acclimatization period during which the shrimp were fed with the formulated diet (FD) at satiation level twice daily (at about 6:00AM and 6:00PM).

Experiment Design

After 24 hours starvation, shrimp with wet weight of 3.0 ± 0.2 g, 8.0 ± 0.5 g, 14.2 ± 0.9 g, and 19.0 ± 1.4 g (Mean ± SD) were selected from acclimated shrimp. For every body weight size, shrimp were fed at 5 feeding levels (0, 0.5%, 1.0%, 1.5% wet body weight, and satiation level) for 28 days. Six groups for every treatment were designed and a complete randomized block design was used to arrange aquaria.

Samples Collection and Analysis

Three groups (8 shrimp each) were sampled from the acclimated shrimp to determine the initial body composition of experimental shrimp. After 28 days all groups of shrimp were starved for 24 hours, and then sampled. The treated shrimp were pooled as three samples randomly (2 shrimp for a sample to get enough material for analyzing) and there were 60 samples (3 per treatment) of final shrimp.

During the course of the experiment the daily food supplied to shrimp was recorded and uneaten food and feces were collected within 3 hours after feeding. Shrimp and food were weighted using an electronic scale after carefully blotted with paper towel to remove excess moisture.

*Corresponding author. E-mail: dongsl@ouc.edu.cn

TABLE 1.

The relationship among the daily growth coefficient (DGC), feeding levels (mg/g shrimp/day), and initial shrimp weight (g).

Model	DGC = B ₀ + B ₁ × FL + B ₂ × IBW (n = 120)					
	B ₀ (Mean ± SE)	B ₁ (Mean ± SE)	B ₂ (Mean ± SE)	R	F	P
DGC _w	-0.236 ± 0.028	0.021 ± 0.002	0.001 ± 0.001	0.780	90.63	<0.01
DGC _d	-1.227 ± 0.059	0.067 ± 0.003	0.024 ± 0.004	0.888	218.94	<0.01
DGC _p	-1.487 ± 0.073	0.078 ± 0.004	0.033 ± 0.005	0.879	198.33	<0.01
DGC _e	-1.611 ± 0.072	0.083 ± 0.004	0.037 ± 0.004	0.893	231.53	<0.01

After weighing all samples of shrimp, feces, and food were dried in an oven at 70° to constant weight, homogenized with a glass mortar, and stored at -20°. Before chemical compositions were analyzed, the samples were re-dried at 70° to constant weight.

Nitrogen content was measured using the MicroKjeldahl methods and the crude protein content was calculated (N × 6.25) (AOAC, 1984). Crude lipid was determined by the Soxhlet method (AOAC, 1984), ash was determined by combusting dry samples in a muffle furnace at 550° for 12 h (AOAC, 1984), and the gross energy content of dry samples was determined with PARR1281 bomb calorimeter (PARR Instrument Company, USA). Analyses of each sample were conducted in triplicate (3 subsamples for each sample).

Calculation of Data

The FE, % was calculated as follow:

$$FE = 100 \times (FBW - IBW) / I$$

Where the shrimp body weight was dry weight (DW, g), the diet was expressed in terms of dry weight (DW, g), FBW was the final shrimp weight, IBW was the initial shrimp weight, I was the quantity of ingested diet during the experiment period.

All samples in terms of dry weight (DW, g), protein (P, g), and energy (E, KJ) were used to calculate the FE (FE_d, FE_p, FE_e) respectively.

The DGC_w was calculated as follow (Cowey 1992):

$$DGC = (FBW^{1/3} - IBW^{1/3}) / D$$

Where D was the days of experiment period. All samples in terms of dry weight (DW, g), protein (P, g), and energy (E, KJ) were used to calculate the DGC (DGC_d, DGC_p, DGC_e) respectively.

The feeding level (mg/g BW/d) was calculated as follow:

$$FL = 2 \times 1000 \times I / (FBW + IBW) / D$$

Items in the energy budget equation (C_e = G_e + F_e + E_e + U_e + R_e) (Klein 1975) of shrimp were calculated as follow:

$$\text{Energy intake (I}_e\text{)} = I_d \times GE_d$$

$$\text{Growth energy (G}_e\text{)} = FS_e - IS_e$$

$$\text{Energy of feces (F}_e\text{)} = F_d \times GE_f$$

$$\text{Energy of exuviae (E}_e\text{)} = E_d \times GE_e$$

$$\text{Energy of excretion (U}_e\text{)} = U_N \times 24.83$$

$$\text{Energy of metabolism (R}_e\text{)} = I_e - G_e - F_e - E_e - U_e$$

Where GE_d, GE_f, and GE_e were gross energy of diet, feces, and exuviations respectively, F_d, E_d, and U_N were dry weight of diet, dry weight of feces and nitrogen of excretion respectively. FS_e and

IS_e were energy content of final shrimp and initial shrimp. 24.83 means the energy cost of 1g nitrogen excreting (in form of NH₃) is 24.83 KJ. The daily unit body weight energy partitions were calculated by dividing 28 × (FBW + IBW)/2.

The nitrogen of excretion (U_N) was calculated as the follow formula (Levine & Sulkin 1979, Lemos and Phan 2001):

$$U_N = I_N - G_N - F_N - E_N$$

Where I_N, G_N, F_N, and E_N were nitrogen content in ingested diets, body growth, feces and exuviations.

The percentage of growth energy (G_e) in energy consumption (C_e) was calculated as 100 × G_e / I_e, and the percentages of F_e, E_e, U_e, and R_e in I_e were calculated similarly.

Statistical Analysis

Statistics were performed using SPSS10.0 statistical software with possible differences among diet treatment being tested by 1-way ANOVA. Tukey's b-multiple range tests were used to test differences between treatment groups of the same size. The square-root transformation of the sine-arc before analyzing the values given in percentages was used. Differences were considered significant at a probability level of 0.05. The curve estimation was used in regression analysis.

RESULTS

Relationship Between Daily Growth Coefficient and Feeding Level

The dualistic linear model (DGC = B₀ + B₁ × IS_w + B₂ × FL) could describe the relationship among the DGC of Chinese shrimp,

TABLE 2.

The relationship between the food conversion and feeding level (mg/g shrimp/day).

Model IBW (g)	FC = B ₀ + B ₁ × lnFL (n = 30)				
	FC	B ₀	B ₁	R ²	P
3.0 ± 0.2	FC _d	-174.2	60.6	0.90	<0.01
	FC _p	-317.3	110.6	0.90	<0.01
	FC _e	-203.5	70.0	0.91	<0.01
8.0 ± 0.5	FC _d	-76.5	29.2	0.65	<0.01
	FC _p	-155.2	57.1	0.72	<0.01
	FC _e	-87.1	32.7	0.66	<0.01
14.2 ± 0.9	FC _d	-146.8	58.1	0.87	<0.01
	FC _p	-217.0	86.1	0.87	<0.01
	FC _e	-155.5	61.1	0.88	<0.01
19.0 ± 1.4	FC _d	-102.6	39.8	0.84	<0.01
	FC _p	-150.0	57.7	0.81	<0.01
	FC _e	-111.6	43.9	0.86	<0.01

TABLE 3.
The energy intake (IE, KJ) and allocation of Chinese shrimp at different feeding levels (mg/g shrimp/day).

IBW	FL	Energy ingested and allocation											
		IE			GE			FE			EE		
		KJ/shrimp	J/gBW/d	%IE	%IE	J/gBW/d	%IE	J/gBW/d	%IE	J/gBW/d	%IE	J/gBW/d	%IE
3.0 ± 0.2	4.4	0.24 ± 0.01	84.7 ± 2.8 ^a	-110.3 ± 6.2 ^a	-92.5 ± 4.7 ^a	13.4 ± 1.6 ^b	11.3 ± 1.3 ^a	12.2 ± 1.4 ^b	10.3 ± 1.2 ^a	166.0 ± 5.1 ^d	18.7 ± 0.9 ^e	140.0 ± 2.6 ^a	15.7 ± 0.4 ^a
	7.9	0.44 ± 0.04	152.1 ± 14.5 ^b	-47.3 ± 8.8 ^b	-67.1 ± 6.6 ^b	9.0 ± 1.0 ^a	13.1 ± 0.4 ^a	10.6 ± 1.4 ^b	15.5 ± 0.9 ^b	117.5 ± 5.8 ^c	10.2 ± 0.7 ^b	175.5 ± 9.0 ^b	15.1 ± 0.5 ^a
	17.7	1.13 ± 0.06	344.8 ± 23.4 ^c	1.2 ± 0.2 ^c	15.8 ± 5.6 ^c	13.8 ± 1.2 ^b	48.1 ± 6.0 ^b	4.7 ± 0.3 ^a	16.7 ± 1.2 ^b	74.4 ± 1.9 ^b	5.9 ± 0.3 ^a	252.8 ± 20.4 ^b	19.6 ± 2.0 ^{ab}
	25.4	1.74 ± 0.15	488.9 ± 36.2 ^d	14.0 ± 2.6 ^d	65.0 ± 11.6 ^d	17.5 ± 1.7 ^b	77.2 ± 6.0 ^c	4.2 ± 0.5 ^a	17.2 ± 1.0 ^b	59.9 ± 3.2 ^a	4.4 ± 0.3 ^a	306.3 ± 30.5 ^c	23.1 ± 2.9 ^b
8.0 ± 0.5	8.7	1.31 ± 0.03	167.4 ± 3.4 ^a	-19.5 ± 2.6 ^a	-32.2 ± 4.1 ^a	13.1 ± 0.4 ^a	21.8 ± 0.5 ^a	8.6 ± 0.8 ^b	14.3 ± 1.3 ^a	88.2 ± 2.4 ^c	9.6 ± 0.4 ^c	147.5 ± 3.9 ^a	16.0 ± 0.7 ^a
	13.1	2.05 ± 0.11	251.5 ± 11.3 ^b	-0.2 ± 0.1 ^b	8.0 ± 0.6 ^b	13.2 ± 3.8 ^a	39.8 ± 2.1 ^b	4.5 ± 0.7 ^a	13.3 ± 0.5 ^a	75.2 ± 3.8 ^b	7.3 ± 0.3 ^b	173.6 ± 11.9 ^a	16.6 ± 1.4 ^a
	17.2	2.82 ± 0.05	331.8 ± 9.5 ^c	10.7 ± 0.8 ^c	33.7 ± 1.9 ^c	14.0 ± 0.5 ^a	45.2 ± 1.4 ^c	3.7 ± 0.2 ^a	11.7 ± 0.9 ^a	65.7 ± 1.2 ^a	5.9 ± 0.2 ^a	221.2 ± 7.6 ^b	20.0 ± 0.8 ^a
	21.2	3.37 ± 0.07	407.5 ± 11.8 ^d	12.1 ± 4.3 ^c	34.8 ± 3.6 ^c	12.8 ± 0.5 ^a	47.7 ± 2.0 ^c	3.5 ± 0.1 ^a	13.4 ± 0.4 ^a	65.6 ± 3.9 ^a	6.0 ± 0.4 ^a	285.2 ± 2.0 ^c	26.3 ± 2.3 ^b
14.2 ± 0.9	4.7	1.22 ± 0.04	90.3 ± 1.7 ^a	-64.1 ± 4.1 ^a	-57.8 ± 3.9 ^a	14.0 ± 0.3 ^a	12.7 ± 0.4 ^a	15.1 ± 0.7 ^c	13.6 ± 0.6 ^a	121.7 ± 3.3 ^c	13.3 ± 0.6 ^c	109.9 ± 3.7 ^a	12.0 ± 0.6 ^a
	9.1	2.45 ± 0.19	175.6 ± 11.9 ^b	-16.3 ± 19.1 ^b	-23.7 ± 7.8 ^b	15.8 ± 0.4 ^a	27.5 ± 1.6 ^b	9.1 ± 1.2 ^b	15.0 ± 0.5 ^a	82.8 ± 4.5 ^b	8.7 ± 0.8 ^b	142.1 ± 6.3 ^b	14.7 ± 0.7 ^{ab}
	12.1	3.34 ± 0.13	233.2 ± 7.4 ^c	-4.5 ± 0.9 ^{bc}	-10.9 ± 6.2 ^c	18.0 ± 1.0 ^b	42.2 ± 3.4 ^c	5.9 ± 0.6 ^{ab}	13.6 ± 1.2 ^a	73.8 ± 1.5 ^b	6.8 ± 0.3 ^a	172.3 ± 7.9 ^c	15.9 ± 1.1 ^b
	14.2	3.89 ± 0.16	272.8 ± 9.1 ^d	2.5 ± 0.8 ^c	6.6 ± 0.9 ^d	23.3 ± 0.8 ^c	63.6 ± 3.5 ^d	5.0 ± 0.8 ^a	13.5 ± 2.1 ^a	63.7 ± 1.4 ^a	5.6 ± 0.2 ^a	173.9 ± 8.3 ^c	15.2 ± 1.0 ^b
19.0 ± 1.4	4.4	1.61 ± 0.04	84.69 ± 1.2 ^a	-44.3 ± 4.2 ^a	-37.4 ± 3.4 ^a	9.4 ± 0.5 ^a	8.0 ± 0.3 ^a	11.3 ± 1.0 ^b	9.5 ± 0.8 ^a	111.5 ± 3.4 ^c	12.0 ± 0.6 ^a	94.4 ± 2.8 ^a	10.2 ± 0.5 ^a
	9.0	3.23 ± 0.56	173.8 ± 25.9 ^b	-22.9 ± 2.0 ^b	-30.8 ± 2.9 ^a	14.1 ± 0.9 ^{bc}	24.2 ± 3.3 ^b	10.8 ± 2.3 ^b	17.1 ± 2.3 ^b	88.6 ± 7.8 ^b	9.5 ± 1.3 ^a	147.9 ± 13.4 ^b	15.4 ± 1.1 ^b
	12.0	4.44 ± 0.10	231.7 ± 4.4 ^c	1.5 ± 0.6 ^c	3.7 ± 0.8 ^b	12.6 ± 0.5 ^b	29.3 ± 1.2 ^b	6.2 ± 0.4 ^a	14.3 ± 0.9 ^b	72.3 ± 1.5 ^a	7.4 ± 0.3 ^b	167.2 ± 3.4 ^{bc}	17.2 ± 0.5 ^{bc}
	14.3	5.43 ± 0.26	279.5 ± 8.2 ^d	2.4 ± 0.6 ^c	5.8 ± 0.9 ^b	14.4 ± 0.5 ^b	40.0 ± 1.6 ^c	5.7 ± 0.6 ^a	15.6 ± 1.4 ^{ab}	70.4 ± 3.2 ^a	7.2 ± 0.5 ^c	197.7 ± 13.7 ^c	20.3 ± 1.8 ^c

Values without same letters in the same parameter of the same body wet weight were significantly different from each other.
The superscript letters a through d in this table were markers of differences of values.

TABLE 4.

Energy loss during experimental period (e_1 , KJ/shrimp) and allocation of starved shrimp (Mean \pm SE).

IBW	Energy loss		R_e		U_e		E_e	
	KJ/shrimp	J/g BW/d	% E_1	J/g BW/d	% E_1	J/g BW/d	% E_1	J/g BW/d
3.0	8.6 \pm 0.4 ^a	173.7 \pm 8.6 ^b	84.0 \pm 1.1 ^b	145.7 \pm 6.7 ^b	12.7 \pm 0.2 ^{bc}	22.0 \pm 1.0 ^b	3.4 \pm 1.3 ^a	6.0 \pm 2.3 ^a
7.9	12.7 \pm 0.5 ^a	171.3 \pm 24.8 ^b	77.1 \pm 2.0 ^a	132.2 \pm 19.7 ^b	13.4 \pm 0.4 ^c	23.0 \pm 3.4 ^b	9.5 \pm 2.3 ^{ab}	16.1 \pm 5.1 ^a
14.5	32.6 \pm 2.0 ^b	99.6 \pm 5.3 ^a	75.6 \pm 1.7 ^a	75.4 \pm 4.7 ^a	10.5 \pm 0.4 ^a	10.5 \pm 0.7 ^a	13.9 \pm 2.0 ^b	13.8 \pm 2.0 ^a
19.1	43.2 \pm 2.8 ^c	101.3 \pm 6.9 ^a	76.7 \pm 1.2 ^a	77.7 \pm 5.6 ^a	12.1 \pm 0.2 ^b	12.3 \pm 0.8 ^a	11.2 \pm 1.3 ^b	11.3 \pm 1.6 ^a

its initial body weight (IBW) and feeding levels (FL) (Table 1). Whatever the shrimp body weight and feeding levels were expressed by wet weight, dry weight, protein or energy, the values of parameter B_1 and B_2 were positive, which means the DGC of shrimp were positively linear correlative to FL and IBW.

The Relationship Between the Food Efficiency and Feeding Level

The FE of Chinese shrimp was significantly correlative to FL, and the linear model $FE = B_0 + B_1 \times \ln FL$ could describe the relationships between FE and $\ln FL$ of the shrimp with four body weight sizes (Table 2). However, with the decreasing of the initial body weight the correlativity (see R^2 in Table 2) was improved.

The Energy Allocation of Chinese Shrimp Fed at Different Feeding

Table 3 denoted the models of energy allocation of Chinese shrimp with different body weight at different feeding levels. The maximal of feeding levels was different in shrimp with different

body weight, and it declined when the body weight rose (Table 3). When the shrimp were fed at the satiation level, the average gross energy intake of single shrimp over the experimental period was 1.74 KJ, 3.37 KJ, 3.89 KJ, and 5.43 KJ respectively for shrimp with different IBW. The fasting energy losses during the experimental period varied from 8.6 KJ to 43.2 KJ according to different sizes (Table 4). The energy loss through respiration (%IE) was greater in small shrimp than that in large shrimp, and energy loss of exuviations (%IE) was greater in large shrimp than that in small shrimp (Table 4). For daily unit body weight energy allocation (J/g BW/d), I_e , G_e , R_e , F_e , and U_e increased as FL increased, regardless of IBW. It seemed that FL did not significantly affected E_e in all IBW.

The Biochemical Composition and Gross Energy Content of Final Shrimp Fed at Different Feeding Levels

The differences of biochemical composition of four size shrimp fed with different feeding levels were listed in Table 5. In the same

TABLE 5.

The body biochemical composition and gross energy of shrimp fed at different feeding levels (mg/g shrimp/day).

IBW	FL	Moisture	Protein	Lipid	Ash	Gross energy
3.0 \pm 0.2	IS	76.9 \pm 0.5 ^a	15.9 \pm 0.4 ^c	1.8 \pm 0.1 ^c	3.2 \pm 0.1 ^a	4.4 \pm 0.1 ^c
	0	87.9 \pm 0.9 ^c	6.8 \pm 0.1 ^a	1.0 \pm 0.1 ^a	3.1 \pm 0.1 ^a	1.9 \pm 0.1 ^a
	4.4	86.8 \pm 0.5 ^{bc}	7.2 \pm 0.1 ^a	1.0 \pm 0.1 ^a	3.4 \pm 0.1 ^a	1.9 \pm 0.1 ^a
	7.9	84.7 \pm 0.8 ^b	11.1 \pm 0.4 ^b	1.1 \pm 0.1 ^a	3.1 \pm 0.1 ^a	2.9 \pm 0.1 ^b
	17.7	78.5 \pm 0.7 ^a	14.6 \pm 1.3 ^c	1.5 \pm 0.1 ^b	3.1 \pm 0.3 ^a	4.0 \pm 0.4 ^c
	25.4	76.1 \pm 0.8 ^a	15.2 \pm 0.6 ^c	2.1 \pm 0.1 ^c	3.0 \pm 0.1 ^a	4.5 \pm 0.2 ^c
8.0 \pm 0.5	IS	78.2 \pm 0.6 ^b	15.0 \pm 0.2 ^{cd}	1.2 \pm 0.1 ^a	3.3 \pm 0.1 ^a	4.0 \pm 0.1 ^{cd}
	0	84.0 \pm 0.6 ^d	10.0 \pm 0.1 ^a	1.0 \pm 0.1 ^a	2.9 \pm 0.1 ^b	2.7 \pm 0.1 ^a
	8.7	80.8 \pm 0.4 ^c	11.8 \pm 0.2 ^b	1.2 \pm 0.1 ^a	3.3 \pm 0.1 ^a	3.3 \pm 0.1 ^b
	13.1	75.9 \pm 0.2 ^{ab}	14.5 \pm 0.1 ^c	1.4 \pm 0.1 ^b	2.5 \pm 0.1 ^d	3.8 \pm 0.1 ^c
	17.2	78.2 \pm 0.6 ^b	16.2 \pm 0.1 ^d	1.5 \pm 0.1 ^b	3.6 \pm 0.1 ^e	4.4 \pm 0.1 ^d
	11.2	75.1 \pm 1.6 ^a	16.3 \pm 0.1 ^c	2.1 \pm 0.1 ^c	2.9 \pm 0.1 ^c	4.7 \pm 0.1 ^e
14.2 \pm 0.09	IS	75.9 \pm 0.6 ^a	15.8 \pm 0.2 ^d	1.8 \pm 0.1 ^c	3.1 \pm 0.1 ^{ab}	4.5 \pm 0.1 ^c
	0	83.7 \pm 1.0 ^b	10.8 \pm 0.1 ^a	0.8 \pm 0.1 ^a	3.2 \pm 0.1 ^b	2.7 \pm 0.1 ^a
	4.7	82.4 \pm 0.6 ^b	11.7 \pm 0.3 ^b	1.0 \pm 0.1 ^b	2.6 \pm 0.1 ^a	3.2 \pm 0.1 ^b
	9.1	77.1 \pm 0.8 ^a	14.9 \pm 0.1 ^c	1.3 \pm 0.1 ^c	3.0 \pm 0.1 ^{ab}	4.0 \pm 0.1 ^c
	12.1	76.8 \pm 0.6 ^a	16.3 \pm 0.1 ^d	1.6 \pm 0.1 ^d	3.2 \pm 0.4 ^b	4.3 \pm 0.1 ^d
	14.2	77.5 \pm 1.0 ^a	17.9 \pm 0.2 ^c	1.8 \pm 0.1 ^c	3.4 \pm 0.2 ^b	4.6 \pm 0.1 ^c
19.0 \pm 1.4	IS	75.9 \pm 1.2 ^a	16.2 \pm 0.2 ^d	1.7 \pm 0.1 ^{bc}	3.0 \pm 0.1 ^c	4.6 \pm 0.1 ^d
	0	85.3 \pm 0.3 ^c	8.8 \pm 0.1 ^a	1.0 \pm 0.1 ^a	3.1 \pm 0.1 ^c	2.4 \pm 0.1 ^a
	4.4	80.5 \pm 0.6 ^b	13.0 \pm 0.3 ^b	1.0 \pm 0.1 ^a	3.2 \pm 0.1 ^d	3.5 \pm 0.1 ^b
	9.0	76.7 \pm 0.9 ^a	13.2 \pm 0.1 ^b	1.4 \pm 0.1 ^b	2.7 \pm 0.1 ^a	3.8 \pm 0.1 ^c
	12.0	78.8 \pm 0.6 ^{ab}	15.7 \pm 0.1 ^c	2.3 \pm 0.1 ^c	2.9 \pm 0.1 ^b	4.5 \pm 0.1 ^d
	14.3	76.8 \pm 1.1 ^a	16.4 \pm 0.1 ^d	2.8 \pm 0.2 ^d	3.0 \pm 0.1 ^c	5.1 \pm 0.1 ^e

IBW, Initial body weight (g); FL, Feeding level (mg/g shrimp/day).

Values without same letters in the same parameter of the same Body wet weight were significantly different from each other.

The superscript letters a through e in this table were markers of differences of values.

TABLE 6.

The relationship between maintainable feeding level (MFL, mg/g shrimp/day) and initial body weight (IBW).

Model		Terms of IBW			
MFL = A ₀ + A ₁ × IBW		Wet weight	Dry weight	Protein	Energy
Parameters	A ₀	11.5	18.3	19.0	19.5
	A ₁	-0.05	-0.36	-0.42	-0.45

size, the body moisture contents of the shrimp fed low feeding level were higher than that of initial shrimp, and it decreased with the increasing of feeding level. The range of the body moisture content of all treatments was from 75.1% to 87.9%. In low FL treatments, the protein content, lipid content and gross energy of shrimp body were significantly lower than those of the initial shrimp, and they increased along with the increasing of feeding level. The body protein content, lipid content and gross energy of four sizes shrimp fed at satiation level (from small to large) were listed below: protein, 15.2%; 19.3%; 17.9%; and 16.4%; lipid, 2.1%; 2.1%; 1.8%; and 2.8%; gross energy, 4.5KJ/g; 4.7 KJ/g; 4.6 KJ/g; and 5.1 KJ/g. It was found that no significant difference existed among different body weight sizes in the protein content, lipid content and gross energy when the shrimp were fed at satiation level.

It was found in the starved and low feeding level treatment that the body moisture content of final shrimp was higher than that of initial shrimp and high feeding level treatment. But the body protein content, lipid content and gross energy of starved and low feeding level treatment were lower than those of initial shrimp and high feeding level treatment. These indicated that the ingested nutrients and energy could not meet the requirement of minimal metabolism in low feeding level treatment, which resulted in the decreasing of body nutrient and energy deposits.

Energy Loss of Fasting Shrimp and Maintainable Feeding Level

It was found that the total energy loss for individual shrimp during the experimental period was directly related to IBW. For unit body weight energy loss per day, the small shrimp (3.0g and 8.0g) lose more energy than large shrimp (14.2 g and 19.0 g). R_e accounted for most of the energy loss regardless of IBW. The relationship between MFL and IBW could be achieved from the relationship between DGC, FL, and IBW (supposed DGC = 0). A linear model was achieved (Table 6) and it inferred that MFL was inversely correlated to IBW. The MIE, (J/g BW/d) for shrimp with different IBW is listed in Table 7. The linear model indicated MIE was also inversely correlated to IBW.

DISCUSSION

Growth Model of Chinese Shrimp

In this study, it was found that the compound relationship among the special growth rate DGC of Chinese shrimp, feeding level (FL) and initial body weight (IBW) were duality linear. It is to say that in every body weight size the DGC and FL were significantly linearly correlated. Similar positive correlations between growth and ration size have been found in studies on shrimp and crabs (Lee 1971, Venkataramiah et al. 1975, Caillouet et al. 1976, Sedgwick 1979, Bartley et al. 1980, Viayaraghavan et al.

1982, Maguire & Leedow 1983, Gu et al. 1996). In this model, when DGC was calculated in wet weight, B_1 was less than one third and B_2 was less than one twentieth of those when DGC calculated in dry weight, protein, or energy (Table 1). This particularity resulted from variability of body moisture content in different conditions. The increase of body moisture content in under-nutritional condition and the reduction of body moisture content in nutrition satiation condition would conceal the alteration of wet body weight.

Effect of Feeding Level on Energy Allocation

In this study, daily unit body weight energy allocation (J/g BW/d) of R_e and U_e were positively correlated to FL regardless of IBW. This result was in agreement with results found in studies on the relationship between metabolic rate or excrete rate and feeding level or ration size in other crustacean (Hewitt & Iving 1990, Du Preez et al. 1992, Burggren et al. 1993, Megaw & Reiber 2000, Peck & Veal 2001). Directly positive relationship existed between G_e and FL. This relationship supported the relationship between DGC and FL. It indicated that the more shrimp ate, the faster the shrimp grew. This was also similar to other studies (Lee 1971, Venkataramiah et al. 1975, Caillouet et al. 1976, Sedgwick 1979, Bartley et al. 1980, Viayaraghavan et al. 1982, Maguire & Leedow 1983, Gu et al. 1996).

Effect of Feeding Level on Body Composition

In this study, starvation and low feeding levels caused increase in body moisture content and reduction in protein, lipid, and energy content of Chinese shrimp, which is in agreement with previous studies on Chinese shrimp (Wu & Dong 2002a, Wu & Dong 2002b) and other species of crustacean (Dall 1974, Cuzon et al. 1980, Barclay 1983, Sasaki et al. 1986, Stuck et al. 1996a, Lin et al. 2004). It could be concluded that starvation and limited food supply would bring similar effects on body composition caused by other environmental stress such as pollution (Wang & Stickle 1988), infection (Stuck et al. 1996b), and nutrition stress (Lim 1997, Stuck et al. 1996a). There was an inverse relationship between body moisture content and growth, whereas the protein, lipid, and energy content were directly related to growth. Similar results have been found in other studies (Stuck et al. 1996a, Lim 1997). Besides, the protein, lipid, and energy content were negatively correlated to moisture content. Weatherley (1981) proposed to form a significant relationship between moisture and other composition such as protein, lipid, and energy in fish. It was easy to determine the moisture content. Then other composition would be easily calculated. But this method is unsuitable for shrimp. Except

TABLE 7.

Maintainable energy intake (MIE, J/g BW/d) for shrimp.

IBW	MIE			
	Wet weight	Dry weight	Protein	Energy
3.0 ± 0.2	218.3	331.1	341.1	349.0
8.0 ± 0.5	213.5	296.5	300.8	305.8
14.2 ± 0.9	207.5	253.6	250.7	252.1
19.0 ± 1.4	202.9	220.4	220.4	210.6

Wet weight, dry weight, protein, and energy means maintain zero wet weight, dry weight, protein, and energy growth or loss.

for nutritional conditions, the smolting cycle also affected the body composition of shrimp (Read & Caulton 1980).

Maintenance Feeding Level of Chinese Shrimp

The metabolic level of unit body weight of animals decline along with the rising of body weight, therefore the MFL of their unit body weight is negatively correlated to their actual body weight. It could be concluded from the values of A_1 that MFL is positively linear correlated to IBW, regardless of terms described in the data. The MEI (J/g BW/d) is inversely related to IBW. It is also a sign of reduction in metabolic level as BW increased. This

fact correlates with results of other studies (Nelson et al. 1977, Emmerson 1985, Dong et al. 1994, Zhang et al. 1998). It was found that maintenance energy intakes (MIE) are higher than unit body weight energy loss of fasting shrimp. For large shrimp, the difference was more obvious. This could attribute to the increase of metabolic rate after ingestion of food and the decrease of unit body weight energy loss.

ACKNOWLEDGMENT

This work was supported by funds from the Project under the Major State Basic Research of China (Grant no. G1999012011) and the State Agriculture Program (Grant no. K2002-16).

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THE ORIGIN OF THE REGULATORY LIMITS FOR PSP AND ASP TOXINS IN SHELLFISH

JOHN C. WEKELL,¹ JOHN HURST² AND KATHI A. LEFEBVRE³

¹NOAA Fisheries, Northwest Fisheries Science Center, Marine Biotoxins Program, 2725 Montlake Blvd. E., Seattle, Washington 98112; ²State of Maine, Department of Marine Resources, Bureau of Resource Management, Shellfish Program, Booth Bay Harbor, Maine; ³NOAA Fisheries, Northwest Fisheries Science Center, Marine Biotoxins Program, 2725 Montlake Blvd. E., Seattle, Washington 98112

ABSTRACT Understandably, commercial and recreational seafood harvesters are interested in how regulatory limits are set for various toxins in seafood. Here we summarize the origins of the safety levels for paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP) toxins. PSP toxins consist of a suite of neurotoxins identified as saxitoxin, gonyautoxin, and their derivatives. The regulatory limit for these toxins (80 µg STX equiv. / 100 g shellfish) was established in the 1930s and is based on bioassays measuring toxic activity in mice. Amnesic shellfish poisoning (ASP) is a more recently discovered syndrome caused by one toxin, domoic acid (DA). It was identified in 1987 and the regulatory limit of 20 µg/DA g tissue was established in the following year, based on the estimated DA dosage levels consumed by the first human victims of ASP. This study attempts to preserve the history of the origin of these regulatory limits, both of which have not changed and have effectively protected consumers of commercial seafood since their implementation.

KEY WORDS: paralytic shellfish poisoning, amnesic shellfish poisoning

INTRODUCTION

At seafood safety meetings, scientists and policy makers are frequently asked by recreational and commercial seafood harvesters about the origins of the regulatory limits set for marine biotoxins in particular seafood's. In the case of paralytic shellfish poisoning (PSP) toxins, the regulatory limit (80 µg/100 g) was established over 60 years ago and its origin is beginning to be lost to the fog of history. Amnesic shellfish poisoning (ASP) caused by domoic acid (DA), on the other hand, is a relatively new syndrome. The first DA poisonings were observed in 1987 in eastern Canada and the regulatory limit for DA in seafood (20 µg/g) was established by Health Canada in the late 1980s. With the discovery of DA in finfish and shellfish on the west coast of the United States in 1991 (Work et al. 1993), the US Food and Drug Administration also adopted the Canadian value.

The regulatory limits for PSP and ASP toxins evolved quite differently. The chemical structures and toxicology of the PSP toxins were only vaguely understood in the 1930s when the limits were developed. In contrast, the chemical structure of DA had already been long established (Takemoto et al. 1966) before its regulatory limit in shellfish was set by Health Canada in 1987. In fact, extracts of marine algae that contained domoic acid and a related congener, kainic acid, had been used as an antihelminthic for children in Japan (Baslow 1969). However, specific information about the toxicity of DA at higher doses in adults was obtained following the first ASP outbreak in 1987. In an effort to preserve the history of the origin of the regulatory limits for PSP and ASP, we have prepared a brief note describing how these values were first derived based on sound epidemiology and toxicology.

Regulatory Limit for PSP Toxins (80 µg STX Equiv. 100 g⁻¹ Shellfish)

The Toxic Syndrome, PSP

PSP is caused primarily by the consumption of molluscan shellfish that have accumulated PSP toxins as a result of filter-feeding on toxic dinoflagellates (Shumway et al. 1988). In some rare cases finfish and crabs have also been implicated as vectors of PSP toxins. Saxitoxin is the most toxic of the PSP toxins (Oshima 1995). At least 16 variants of this basic structure have been iden-

tified and the variants range in toxicity from high (saxitoxin) to almost nontoxic (the sulfonated toxins). The composition of the suite of PSP toxins found in shellfish is both dependent on the specific phytoplankton (*Alexandrium* sp.) consumed and the metabolic processes within the organism, since shellfish may convert one toxin to another. A full discussion of this molluscan conversion process is beyond the scope of this study, but a detailed description can be found in Sullivan et al. (1983). Thus, PSP is caused by a "suite" of toxins present in shellfish. The syndrome is characterized in its most severe form by paralysis of the breathing muscles, which if left untreated by competent medical intervention, ultimately leads to death. In cases of paralysis, mechanical ventilation is highly effective. However, at lower doses, symptoms can range from mild stomach upset to a tingling sensation in the lips.

Alcoholic Versus Acidic Extraction Methods for PSP Toxins

In 1937, Sommer and colleagues established the connection between toxic shellfish and blooms of the dinoflagellate *Alexandrium catenella* (then known as *Gonyaulax catenella*) along the California coast. Sommer and Meyer (1937), in the course of their monumental study on PSP toxins, developed two extraction methods in their attempts of quantifying the amount of toxin present in shellfish. Their preferred method used acidified alcohol for the extraction of the toxin. Due to the laborious nature of this method, they developed a second more practical "field test" that used diluted hydrochloric acid and boiling for extraction. This field assay also used mice for the quantification of PSP toxins. Whereas the alcoholic extraction has been largely forgotten, the simplified field test (which was only minimally described) is still used today, with minor modifications, as part of the risk management of PSP throughout the world. Because the chemical structure and properties of the shellfish toxins were unknown in the 1930s and the purity of the extracts were also unknown, Sommer and Meyer's field test required them to define toxicity in terms of a Mouse Unit (MU) to quantify toxic activity. In their original work, Sommer and Meyer defined the mouse unit as the amount of toxin that killed a 20 g mouse within 10 to 20 min.

Sommer and Meyer's field method of extraction called for the

homogenization of 100 g of shellfish with 100 mL of 0.1 N hydrochloric acid, boiling this mixture, adjusting the final volume to 200 mL, and then filtering the mixture. Testing for toxin required the intraperitoneal injection of 1.0 mL of the filtrate into a mouse and then observing how long it took the mouse to die; therefore, an injection that killed a mouse in 15 min contained, by definition, 1 MU, and the total toxin content of the original 200 mL extract was 1.0 MU/mL \times 200 mL of extract or 200 MU. Because this extract was obtained from 100 g of shellfish meats, the shellfish were said to contain 200 MU per 100 g of shellfish.

Purification of STX From Shellfish

This unit of measuring PSP toxins was used up until the 1950s when a purified toxin standard became available from the US Food and Drug Administration (FDA). In the late 1940s and 1950s, researchers at Fort Dietrich isolated and purified saxitoxin (STX), the principal toxic component of PSP. Whereas initially intended for chemical warfare purposes, the unused portion of STX was eventually released to the FDA and became a shellfish standard. An official method that used this standard as a reference was accepted by the AOAC in 1959 (McFarren 1959). To use this standard as a control in bioassays, it was necessary to establish a relationship with the MU. Through experimentation, it was found that one MU was equal to approximately 0.2 μ g of the purified FDA standard, although variability between mouse strains ranged from 0.17 μ g to 0.28 μ g (J. Wekell unpub data). Because of this variation between strains, laboratories performing the mouse bioassay must "calibrate" their particular mouse strain against this toxin standard.

Calibration of the Mouse Unit

When the FDA accepted the use of the standard, they also accepted that 0.2 μ g STX equiv. = 1 MU as a reasonable average for a conversion factor (CF). Therefore, 200 MU became equivalent to 40 μ g of the purified toxin. Because the 200 MU detection limit was established from a 100 g batch of shellfish, the detection limit was subsequently expressed as 40 μ g STX equiv./100 g of shellfish, which remains the value that is still used today. Most likely, if the detection limit were set today, it would be expressed as 0.4 μ g/g or 0.4 ppm. However, because virtually all of the literature and databases have used the " μ g/100 g" notation for more than 60 years, it continues to be used to this day.

The use of a purified toxin standard also permitted more consistent estimates of PSP in shellfish. Sommer and Meyer developed a curve plotting MU and "death-times" (Fig. 1A). The curve in Figure 1 inset was derived from data in the report by McFarren (1959) and is currently used by regulatory agencies (AOAC 2002). Examining the death time curve, it is clear that very fast death times (e.g., less than ~5 min) can lead to very large errors in obtaining the correct number of MU. For this reason, the current regulatory method requires dilutions of the sample to bring the death times of the mice within 5 to 7 min, an area where the curve has a very minimal slope and time can be measured with reasonable precision and also permit good interpolation from the curve (Fig. 1B). From the curve and assuming a mouse correction factor of 0.2 we can derive the following: mice dying sooner than 5 min would exceed the regulatory limit of 80 μ g/100 g. Death times greater than 15 min place the concentration of PSP toxins at 40 μ g/100 g, approximately the accepted detection limit of the mouse bioassay. Therefore, using the mouse bioassay a regulatory labo-

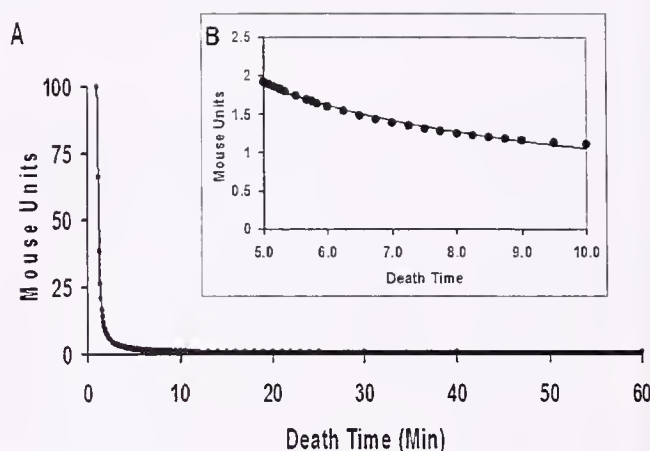


Figure 1. Death times plotted against mouse units (A) and the suggested working range for the mouse bioassay (inset, B). Data from McFarren (1959) and AOAC (2002).

ratory can know in less than 10 min of injection whether dangerous levels of toxin are present in the shellfish.

New Chemical Techniques for Characterizing the Structure of Toxins

Within the past 30 years new chemical techniques such as high performance liquid chromatography (HPLC), x-ray crystallography, and nuclear magnetic resonance spectroscopy (NMR) have permitted the elucidation of the structure of the PSP toxins. The predominant toxin was named saxitoxin after the origin of the initial toxin source, the Alaska butter clam (*Saxidomus giganteus*). A trace component in the FDA standard was later found to be a close chemically related structure and was named neosaxitoxin. Using the techniques listed earlier, further research revealed that PSP poison found in shellfish consists not only of saxitoxin and neosaxitoxin but also a suite of closely related toxins that are commonly referred to as "gonyautoxins" (named after the original genus of the dinoflagellate that produces the toxins). Subsequent studies have shown that not all of these related toxins are equally toxic to mice or humans; some are more toxic than others. Because there is only the one toxin standard, predominantly saxitoxin, the resulting collective concentration of toxins in shellfish extracts are referred to as "X μ g saxitoxin equivalents/100 g shellfish"; sometimes abbreviated as " μ g STX equiv/100 g".

Establishment of the Regulatory Limit (80 μ g STX Equiv. 100 g⁻¹ Shellfish)

How the specific 80 μ g 100 g⁻¹ regulatory level was arrived at is open to some conjecture and the details are now, after over 60 years, probably lost to history. Prior to the establishment of the acid extraction method (only briefly described in Sommer & Meyer 1937) as the standard for regulatory purposes, California instituted quarantine measures when 2 mg of dried alcoholic extract contained 2 MU (Medcof et al. 1947). Tests at the Laboratory of Hygiene have shown this 2 mg to be equivalent to a toxicity of 400 MU per 100 g of whole meats, which is 80 μ g 100 g⁻¹ in today's parlance (Medcof et al. 1947). Canada also adopted the California standard of 400 MU as the quarantine level (Medcof et al. 1947).

Medcof et al. (1947) also examined epidemiologic records from

eastern Canada in the mid 1940s and found that in some cases a dose of 1,000 MU produced mild symptoms of PSP. Using the current conversion factor, 1,000 MU equals approximately 200 μg of STX equivalents, because the lowest dose reported for illness is 200 μg , and assuming that 100 g of shellfish meats might represent a reasonable quantity for an adult to consume, the lowest illness-producing concentration in shellfish would be 200 μg STX 100 g^{-1} . Based on these estimates and using a $\times 10$ safety margin, the regulatory level could be set at 20 μg 100 g^{-1} . However, this is well below the mouse bioassay detection limit. Therefore, the 80 μg 100 g^{-1} level was probably derived as a compromise based on the detection limit of the mouse bioassay (roughly 40 μg 100 g^{-1}) and yet still safely removed from the minimal toxicity of 200 μg 100 g^{-1} observed in the early studies.

Prudence and caution by risk management agencies over the years have maintained the regulatory level at 80 $\mu\text{g}/100$ g. This level seems to have weathered the "test of time" in that after 60 years of use the authors are not aware of a case of PSP reported in properly tested and released shellfish. Virtually all recent illnesses and deaths due to PSP are due to the recreational or subsistence victims ingesting untested shellfish or shellfish taken from quarantined or untested beach areas. In Alaska, because of the remoteness of the beaches and shoreline, the state has mandated that all beaches are quarantined to the taking of all shellfish unless otherwise specified by the state health authorities.

Regulatory Limit for Domoic Acid (20 μg DA/ g Tissue)

The Syndrome, ASP

Domoic acid (DA), the toxin responsible for amnesic shellfish poisoning (ASP), is a recent arrival to the list of known marine biotoxins and is naturally produced by some diatoms of the genus *Pseudo-nitzschia*. However, not all members of the *Pseudo-nitzschia* genus are highly toxigenic. The first reported ASP event occurred in Eastern Canada in 1987 when 4 people died and over 100 people suffered varying degrees of intoxication after consuming DA-contaminated mussels taken from Prince Edward Island (Todd 1990). Herbivorous fish were the vectors of DA in more recent intoxication episodes along the west coast of the United States in which dozens of sea birds and marine mammals died or were stricken with neurologic symptoms (Work et al. 1993, Lefebvre et al. 1999, Scholin et al. 2000). Fortunately, due to an extensive monitoring and surveillance program by the western coastal states (California, Oregon, Washington, and Arkansas) and the Province of British Columbia, no human cases of DA poisoning have been reported and officially confirmed since 1987.

Domoic acid is a low molecular weight, neuroexcitatory amino acid that can cross the blood-brain barrier and destroy neural brain cells. It attacks the glutamate signaling nerve system, destroying the cells that release the neurotransmitter glutamic acid (Debonnel et al. 1989, Berman & Murray 1997). Because regeneration of most brain cells is a relatively slow process, it appears that for practical purposes this damage is permanent and irreversible. In the known cases so far, elderly people seem to be more at risk than younger people (Todd 1993). In the Canadian outbreak, some people were institutionalized with permanent brain damage, while others have suffered varying degrees of short-term memory loss (Todd 1990, 1993, 1997). Follow up studies of the 1987 victims reported that some lost their businesses and the ability to conduct normal daily personal affairs. There is no known treatment for this intoxication.

Establishment of the Regulatory Limit for DA (20 μg DA/ g Tissue)

As in the case of PSP, arriving at a toxic dose of domoic acid in the 1987 episode was difficult because it was dependent largely on indirect measurements on recovered or uneaten products. Two important workshops that strived to set regulatory limits for DA were held immediately after significant DA outbreaks: The first was held in Ottawa, Canada from April 11 and 12, 1989 (Proceedings of a Symposium, Domoic Acid Toxicity, Can. Diseases Weekly Report, Vol. 16S1E, September 1990) and the other was held in San Pedro CA, February 6 to 8, 1992 (Domoic Acid Workshop, San Pedro, California, US Food and Drug Administration, San Francisco, CA, 1991). Lengthy discussions during these workshops illustrate the difficulty of arriving at the most rational regulatory levels that balance the protection of public safety with the economic provision of shellfish resources.

Because no documented and certified human illnesses were ever reported during the 1991 and 1992 United States west coast DA outbreak, all consumption and dose analyses were based on 1987 Canadian data. Recovery of uneaten mussels involved in that outbreak and subsequent analysis indicated that they contained levels ranging from 310 ppm to 1280 ppm domoic acid (Perl et al. 1990). Using these data, it was concluded that victims exhibiting mild and severe symptoms ate shellfish containing approximately 900 ppm DA and received a total maximum dose of between 200 to 300 mg (Wright et al. 1990). The lowest total dose that a minimally symptomatic victim received in the Canadian outbreak was reported to be about 50 mg (Iverson et al. 1989, Hynie et al. 1990, Iverson & Truelove 1994). Assuming a human adult of 70 kg, this translates into a toxicity range of 0.7 mg to 4 mg/kg, very close to animal oral toxicity levels in rats, mice, and monkeys (Iverson et al. 1990); however, mice and rats seem to be less sensitive than primates to the effects of DA. Using the lowest total dosage of 50 mg, assuming a consumption of 200 g shellfish meats, and a safety factor of approximately 12, it was concluded that the regulatory limit should be set at 20 μg DA g^{-1} shellfish.

In 1992, at a Domoic Acid Workshop held in San Pedro, CA sponsored by the FDA, the toxicity data was reexamined by epidemiologists and toxicologists from Canada and the FDA. During the San Pedro workshop, it was suggested that consumption of certain shellfish might be higher than 100–200 g of meat, perhaps as high as 500–1000 g. Nevertheless, after an extended discussion that reviewed the Canadian data, it was concluded that setting the regulatory limit of 20 μg DA g^{-1} shellfish would provide sufficient safety margins that would account for the variability between individual responses and the amount of shellfish consumed.

SUMMARY

The regulatory limits for both ASP (20 μg DA \bullet g^{-1} shellfish) and PSP (80 μg STX equiv. \bullet 100 g^{-1} shellfish) toxins have not changed since they were first implemented. These safety levels seem to be successfully protecting the seafood-consuming public and since their establishment no human cases of ASP or PSP intoxication resulting from regulated commercial seafood have been reported. These levels were driven by the detection capabili-

¹The San Pedro Workshop publication by the Pacific Region FDA office in San Francisco is a compilation of transcriptions of the semi-formal talks and discussions of the participants. Other than minor editing of typed transcripts, no formal peer review of the presentations was made.

ties of the time and the necessity to be conservative in the face of sketchy epidemiologic and toxicologic data. However, the value of this work, done up to 60 years ago in the case of PSP, is exemplified by quite literally "standing the test of time". Those levels, set in the 1930s, are still used today with only minor modification to the assay method itself.

Of course all regulatory risk managers would like to reduce risk to zero, but they know that this is not possible in the real world.

The management of marine toxins is a balance between a reasonable level of safety and complete closure/embargo of all seafood products. Considering the wide diversity of marine phytoplankton species, the number and variety of harmful marine natural products must also be significant. The development of reasonable safety guidelines is complicated by our limited knowledge of the known marine biotoxins, particularly how, when, where, and why harmful algae produce them.

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COVER PHOTO: Geoducks (*Panopea abrupta*) harvested off West Coast Vancouver Island, British Columbia. Photo courtesy of Grant Dovey, Underwater Harvesters Association, Ladysmith, B.C., Canada.

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JOURNAL OF SHELLFISH RESEARCH

VOLUME 23, NUMBER 4

DECEMBER 2004



The Journal of Shellfish Research
(formerly *Proceedings of the National Shellfisheries Association*)
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Journal of Shellfish Research

Volume 23, Number 4

ISSN: 0730-8000

December 2004

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5th International Symposium on Abalone Biology, Fisheries, and Culture

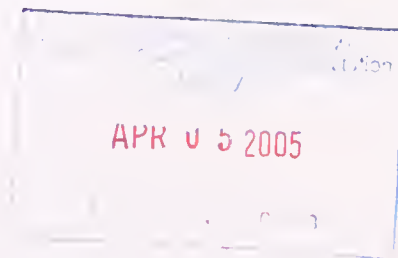
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PREFACE

The 5th International Symposium on Abalone Biology, Fisheries and Culture was held at the Ocean University of China, Qingdao, China, from October 12–17, 2003. The original intention was to hold the symposium in April of 2003 but, because of worries about the SARS epidemic, the event was postponed for about 6 months, until the major concerns about the epidemic had passed. Despite some lingering apprehension, the conference attracted almost 300 delegates from 20 different countries, including representatives from the Americas, Africa, Asia, Oceania, and Europe. This Special Issue of the *Journal of Shellfish Research* contains many of the contributions presented at the conference.

The tradition of holding international abalone symposia started in La Paz, Mexico in 1989. At that time, symposia were held at roughly three-year intervals, in Hobart (Tasmania), Monterey (California), Cape Town (South Africa) and Qingdao (China). All of these symposia were extremely successful, and the number of people attending has gradually increased. Judging from the extensive list of delegates attending the Qingdao symposium, it is obvious that interest in abalone biology, fisheries, and aquaculture is still increasing.

The prominence of abalone in international seafood trade is well known. The astronomical prices paid for it, in certain international markets, is legendary. No wonder then, that abalone aquaculture production is rapidly increasing. The downside, however, is that worldwide fisheries are generally declining, not least because of the fact that the international illegal trade is still unac-

ceptably high. Despite regular calls to governments for actions to be taken against the illegal traders, poaching is still bringing some abalone populations to the verge of extinction. It is partly through the highlighting of this situation, through international symposia, that the plight of the world's abalone populations can be brought to the attention of politicians, hopefully encouraging them to take action.

The Qingdao symposium provided a platform for the presentation of well over 100 oral presentations and about 60 posters. Topics ranged widely and included international production and marketing; genetics; physiology and molecular biology; nutrition; diseases, fisheries and ecology; and aquaculture methodologies. This volume contains a selection of papers from each section, and all papers have been through the usual rigorous refereeing and review procedures of the journal. I wish to thank all authors who submitted manuscripts for publication and, in addition, I wish to thank the numerous referees who volunteered their time to review the papers. In particular, it is a pleasure to see the many papers that were submitted by Chinese authors for whom translation into English is often an additional burden to the presentation of their science.

The 6th International Abalone Symposium will be held in Chile in 2006.

Peter Cook
President, International Abalone Society

WORLD ABALONE FISHERIES AND AQUACULTURE UPDATE: SUPPLY AND MARKET DYNAMICS

H. ROY GORDON¹* AND PETER A. COOK²

¹*Fishitech Inc., Box 6886, San Rafael, California 94903;* ²*Center of Excellence in National Resource Management, University of Western Australia, Albany, 6330, Australia*

ABSTRACT Culminating in the year 2002, a global shift in both abalone availability and distribution occurred. Country by country abalone reporting by FAO for both wild caught and cultured abalone changes significantly when further dissected and standardized. The FAO practice of reporting totals for “abalone shucked or not” and the continuing practice of some countries that still report their production by large groups of species, distorts both historical and current totals. Further review shows that the overall world supply of abalone, as a result of continuing increases in cultured production, combined with an unfortunate continued increase in the illegal wild catch is approaching the historical abalone abundance of the mid 1970s. However, major shifts have occurred when these dynamic changes are analyzed. These significant shifts demand inventive thinking on the supply side if prices are to remain at strong levels. Examples are given of approaches employing new creativity in the world of abalone marketing, distribution, and processing.

KEY WORDS: abalone, abalone supply, abalone fisheries, cultured abalone, abalone prices, abalone marketing

INTRODUCTION

An understanding of the dynamic changes in the world's abalone supply requires a comparison of the decade of the 1970s with the most current year: 2002. The abalone world of the 1970s was one with minimal regulation of the fisheries sector, little illegal catch, and a cultured “market size” industry measuring production in kilograms, not tons. In sharp contrast is the year 2002: 2 major abalone fishery countries (United States and South Africa) have either closed the fishery entirely or have threatened closure. During this same 3-y period the illegal catch has run rampant to the point where it now approaches 40% of the total legal fisheries. Nonetheless, much of the fisheries shortfall continues to be replaced through abalone farming. These factors lead us to the conclusion that the world's supply of abalone (fisheries, cultured, and illegal catch) in 2002 exceeded what was considered the “heyday” of world abalone supply during the 1970s. This surprising return to substantial supply quantities (in which the illegal catch plays no small role) suggests that substantial brand identification, specialty processing, and other marketing efforts/innovations are needed.

As a background reference, the following definitions are repeated (Gordon & Cook 2000):

Abalone fisheries: The total allowable annual commercial landing quota (country by country). This category includes the planting of seeds in large areas of the sea wherein the sea bottom has not been prepared with man placed rocks or structures. This definition does not include the legal sport catch or any illegal catch worldwide.

Cultured abalone: Includes both the farming of abalone on land or sea—contained in man made tanks, nets or structures (intensive culture), and in-sea planting of abalone seeds in artificially arranged substrate or structures, with or without adding food (extensive culture).

Illegal catch: Any world harvest of abalone beyond the total allowable annual landing quotas.

ABALONE FISHERIES

It is well known that over fishing, disease, habitat loss, and failed governing body management of the illegal catch have all

contributed to the decline of the abalone fisheries catch over the past 3 decades. There has been improvement in all but one of these factors (the illegal catch). Figure 1 illustrates country by country fisheries landings for the year 2002 as compared with low to high range averages for abalone fishery countries in the decade following 1970 (FAO historical data 1970/1980)

THE ILLEGAL CATCH

Certainly there was some illegal catch of abalone 2–3 decades ago; however it pales in comparison with the massive illegal take from the late 1990s to the present. Despite a myriad of laws and penalties in many countries, the illegal catch continued in 2002 at an unprecedented pace (Fig. 2). The relationship of the market price to ease of capture created a criminal market (in major producing countries they are well organized, well financed, and often armed). The entire United States abalone fishery was closed indefinitely in 1997, yet the illegal catch continues at over 120 mt per year (California Department of Fish and Game 2002). South Africa's law-enforcement authorities have been engaged in an armed battle to stop that country's illegal catch for a number of years. South African police (SAPS) 6-mo figures show 431 arrests between August 2002 and January 2003, with the admission that even with this strong effort “a lot of abalone are getting past us” (Groenewald 2003). For the year 2003, the SA Fisheries Department considered closing down any form of abalone harvesting once current rights expired. It was estimated that if poaching continued at the current rate, abalone would be fished to extinction in less than 3 y (Duvnhage 2002). The situation is so acute that for the year 2002 the illegal catch was estimated at more than double the 350 mt landings of authorized permit holders. (Tarr 2002).

The Illegal catch in other abalone producing nations is substantial with deep-felt concern in Australia, Mexico, New Zealand, and Japan. There is also a notable illegal catch in other abalone fisheries (Korea, Philippines, Taiwan, and other Indo-Pacific countries).

CULTURED ABALONE PRODUCTION

Figure 3 illustrates the total cultured production as reported within each country (see Acknowledgments). The phenomenal growth of the world cultured abalone is evident when comparing the 8,696 mt for the year 2002 with just 689 mt 15 y earlier (as

*Corresponding author. E-mail: pacook@agric.wa.gov.au

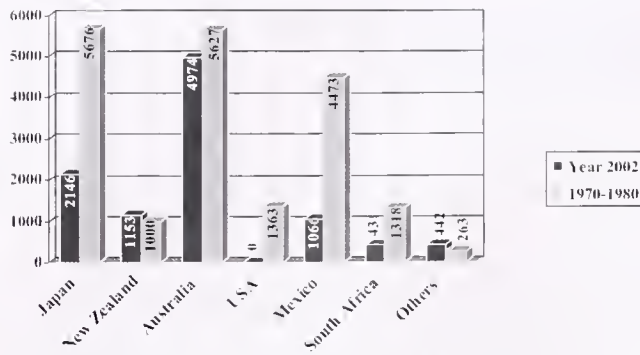


Figure 1. World fisheries abalone landings (Illegal catch not included).

reported the first International Symposium on Abalone Biology, Fisheries and Culture, La Paz Mexico November 1989). Worldwide, more than 15 species are now being commercially cultivated with over 1,000 individual farms with individual production ranging from less than a ton to over 200 mt.

WORLD SUPPLY OF ABALONE FROM ALL SOURCES

In Figure 4 we compare the 2002 worldwide total of abalone from all sources (fisheries landings, illegal catch, and cultured production) with the abundant supplies of the 1970s; we find a near parity. There are however some substantial species differences: most notably lesser quantities of *H. rufescens*, *H. fulgens*, and exceptionally large quantities *H. diversicolor supertexta*, and to a lesser extent, *H. asinina*, and *H. ovina* (Nie 2002)

INDUSTRY CHOICE—GENERIC SELLING OR MARKETING?

Figure 5 represents a rather typical newspaper advertisement (in this case a "supermarket" in Hong Kong). Referring to footnote numbers we have superimposed onto the ad: "1" is frozen abalone from South Africa and "6" Frozen Green Lip from Australia. The ad merely sells frozen abalone from South Africa and Australia at the same price: there is no description of location within the country; there are no brand names, and no reference as to the quality of the supplier's preparation process. Because some older buyers have biases as to one species or another, others would pay more for further guidance other than country of origin and "frozen". The Supermarket's suppliers by default become "generic" (i.e., the sellers to the supermarket were merely selling frozen abalone) with

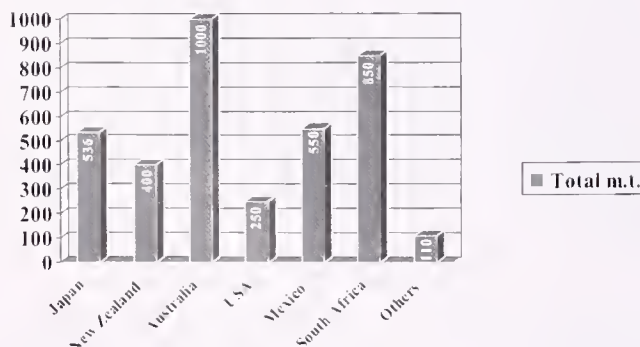


Figure 2. World illegal abalone catch.

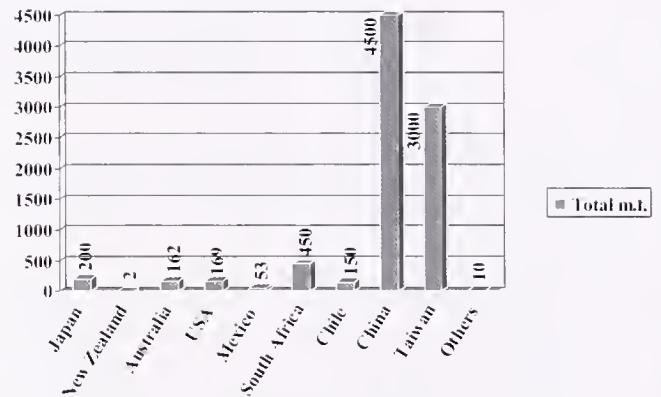


Figure 3. 2002 world cultured abalone production.

little attempt at well deserved and meaningful individual supplier or cooperative supplier marketing effort.

Contrast this with the marketing effort (Fig. 6) showing the cover of a processors brochure with brand name directly appealing to Asian markets. Inside the brochure are references to the specific waters and other perceived advantages of purchasing this brand name whether fresh, frozen, or canned. This strategy will, over time, result in continuing higher profits for this processor.

Figure 5 also identifies: "2" New Zealand canned, "4" CalMex brand from Mexico, and "5" canned from Australia. Canned abalone of necessity have some "brand" name on the can label as is the case "2" and "5", however the price of "2" and "5" are in the same range, whereas CalMex "4", with only slightly higher net weight commands, nearly double the price—an example of strong brand name marketing and recognition.

There is further marketing opportunity in specialty processing/preparation of abalone in any of its various forms. Figure 7 dis-

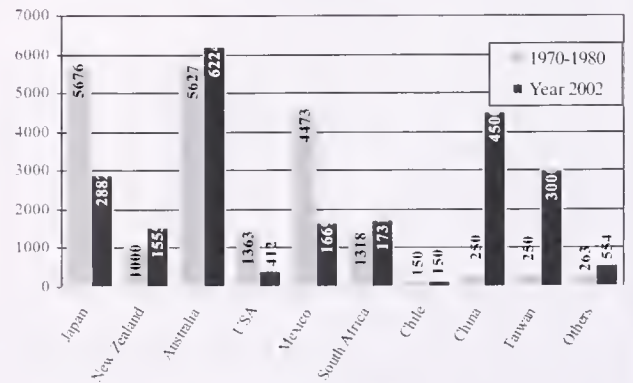
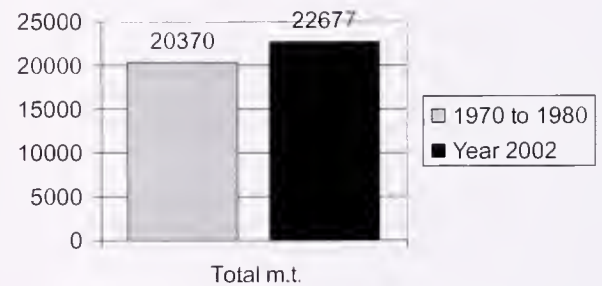


Figure 4. World supply of abalone from all sources.



The downturn in some Asian economies and, in particular Ja-

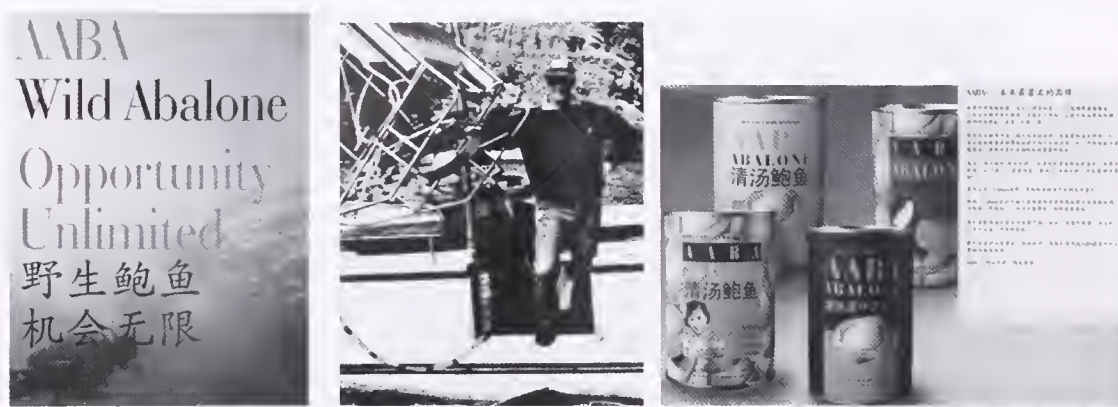


Figure 6. Fisheries branding example.

pan, has certainly played a role in this downward price pressure; however a powerful factor has been the huge illegal fisheries sales into the world marketplace.

CONCLUSIONS

The abalone industry is currently suffering from the proliferation of the illegal catch. Unless governmental enforcement is substantially increased, the continued taking of these often immature animals will irreparably endanger the resource. This illegal catch also applies an important downward pressure on abalone prices because these abalone are most often sold in "peculiar" channels at well under commercial market prices. The slow recovering economy in parts of Asia also plays a role in suppressing prices. Nevertheless, whether considering the fisheries or the cultured industry, abalone in most all of its forms remains considerably under priced for this "shellfish caviar" product. The time has long

since come for those willing to put forth the effort to fully understand the current and new distribution chains (in particular China) and specific target distributors, wholesalers, and mega end users (DWEs). Next comes needed effort in processing and branding with realistic product marketing programs, both on an individual company basis and through "yet to be developed" abalone marketing co-operatives.

ACKNOWLEDGMENTS

Contributions were made by: Tomohiko Kawamura (Japan), Nie Zong Qing (China), Arthur Seavey and the California Aquaculture Association (USA); Enrique Vazquez Moren (Mexico), Cristobal Borda (Chile), Steve Edwards and Steven Reynolds (Australia); Rodney Roberts (New Zealand); Peter Cook (South Africa); Terrence O'Carroll (Ireland and Europe); and Alawai Salim Al-Hafdh (Oman).



Figure 7. Japanese gift catalog.

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THE STATUS OF ABALONE CULTURE IN CHINA

ZONGQING NIE* AND SUPING WANG

Fuzhou Fisheries Research Institute, 3 Gule Rd., Fuzhou 350005 People's Republic of China

ABSTRACT Since 1990 abalone culture has spread widely from north to south on China's coast. Estimated total yield for the year 2001 was around 4,500 tonnes (excluding Taiwan). There are several hundreds of abalone farms along the coast. Some of the bigger farms produce more than 100 t of live abalone each year. The main cultured species are *Haliotis diversicolor aquatilis* Reeve in the south and *H. discus hannai* Ino in the north. Additionally *H. discus discus* Reeve has been introduced from Japan and hybridized with *H. discus hannai*. Crossbreeding has been applied in production. Tank culture and floating raft methods are important in the south and north respectively. At present, epidemic virus diseases and *Vibrio* are the gravest threat. Farms have suffered heavy economic losses as a result of these.

KEY WORDS: abalone, culture, China

INTRODUCTION

Since the previous review of abalone culture in China, written for the 1989 First International Symposium on Abalone Biology, Fisheries and Culture (Nie 1992) there has been dramatic change. Abalone culture in a mass scale in China began in the mid 1980s. It developed rapidly in the 1990s spreading along the coasts from north to south. Estimated abalone output was about 4,500 tonnes (excluding Taiwan) in 2001, 99% produced by cultivation. *Haliotis diversicolor aquatilis* (i.e., *H. diversicolor supertexta*) was around 65% of the total yield. The cultured species are varied. The technique of crossbreeding has been used widely (Nie et al. 1995a, Nie & Wang 2000). Triploid breeding has already started in production situations (Wang et al. 1990, Sun et al. 1993, Sun et al. 1998, Zhang et al. 1998). Because the abalone industry has developed rapidly sales prices have declined sharply. This has been especially so for the price of small abalone. For example *H. diversicolor aquatilis* sales price has been sometimes less than the cost of production. Epidemic diseases have occurred frequently since 1993 (Liu et al. 1995, Nie et al. 1995a, Ma et al. 1996). In particular a virus disease has appeared successively during late winter in the south since 1999 (Huang et al. 1999). Farms have suffered heavy losses and the virus is a serious threat for abalone culture at present.

Cultured Species and Distribution

There are eight species of abalone along China's coasts. Geographically *H. discus hannai* Ino is the only species in Liaoning and Shandong Provinces in northern China. The other seven species are distributed in Fujian, Taiwan, Guangdong, and Hainan, the southern provinces. These species are *H. diversicolor diversicolor* Reeve, *H. diversicolor aquatilis* Reeve, *H. varia* Linnaeus, *H. planata* Sowerby, *H. ovina* Gmelin, *H. clathrata* Reeve, and *H. asinina* Linnaeus (Lu 1978, Dai & Wu 1989). Because *H. diversicolor diversicolor* grows slowly, *H. diversicolor aquatilis* becomes very important. This species was introduced from Taiwan to the southern continental coasts in 1989 and is now raised widely there. Additionally *H. discus discus* Reeve was introduced from Japan in 1986, *H. discus hannai* Ino from Dalian in 1990 and their hybridized generation are raised in the mid and northern Fujian (Nie et al. 1995b). There are now several hundreds of abalone

farms in China. The largest produce more than 100 tonnes of live abalone per year.

Culture Methods

The culture methods used in China are quite varied. Because of the coastal and climatic conditions they are different in the north and south. Long line raft is an important method in the north. A length of 60–80 m of rope ties together plastic floating globes to support cages. There are two sorts of cage currently in use. A special cage for abalone culture consists of 6–12 tiers of 60-cm diameter plastic dishes. The entire assembly is covered with a net sealed with a zipper. The second type used is a scallop cage, of 8–12 tiers of 33-cm diameter. Most rafts are mixed culture with abalone and algal food, *Laminaria* or *Undaria*. Rafts are also used in some bays in the south where the wind and waves are weak. In these instances a plastic basket, the same as indoor tank culture baskets, is used instead of the other cages.

Tank culture on land is the main type in the south. Because of frequent typhoons the culture had to develop on land. Concrete tanks are used of around 30–50 cubic meters. A dark PVC basket of 40 cm × 30 cm × 13 cm is in common use, usually in a stack of 8–12 tiers arranged in rows in a tank. The baskets occupy 40–70% of the tank capacity (Nie & Wang 2000).

In the western Guangdong and Hainan Province, in recent years, small abalone are cultured in the intertidal zone. A type of cylindrical concrete case, 110 cm × 80–120 cm (h) with an iron rack and net inside, is set on a low intertidal zone (Cai & Huang 2000).

Rocky intertidal zones in the north are also used. Here growers build frames and cover with net as well as other types of cages (Zhang et al. 1994).

Breeding Techniques and Present Problems

There are some differences in breeding techniques. The methods in the north are similar to Japanese methods in many ways, and in the south the methods approximate those of Taiwan.

All hatcheries in the north maintain parent stock with elevated temperature (20°C) in winter and induce spawning in spring. This allows sufficient warm weather for growing larger seed (to >15 mm) before the coming winter. In contrast the small abalone can induce spawning naturally from May to December (main season is September to November) in the south. Nonetheless some farms in the south also maintain parent animals with elevated temperature in winter and induce spawning from April to June.

*Corresponding author.

The substrate for larval settlement in the north is a corrugated plastic plate (40 cm × 31 cm) held in racks. Variations appear in the southern farms. Most farms prefer a transparent plastic sheet (60 cm × 40 cm) frequently set up so that many plastic sheets are hung under a wooden bar. Other types of membrane (rather than racked) corrugated plates are also used (Nie & Wang 2000).

A method of spawning induction with UV light and water temperature alteration (3°C to 5°C) is current. In the north, the fertilized eggs are maintained to the post veliger stage and then put into a tank for settlement. In the south it is more common to place fertilized eggs directly in a settling tank, with no water exchange until larval settlement. From the viewpoint of mass production the latter is quite feasible because it saves a lot of labor, time, and facilities.

The young juveniles are removed from the transparent plate/membrane at 2–4 mm, a much smaller size than during the early years of abalone culture in China. Commonly, in the north, the juveniles are then transferred in a box net with a dark plate on the net bottom. In the south they are raised directly on the bottom of another settling tank, on a substrate of square bricks with short legs that are arranged across the bottom of the settling tanks. The density of rearing is around 3,000–5,000 juveniles per square meter. A survival rate of about 40% to 60% is obtained and measured from the removal phase (2–4 mm) up to the end of nursery rearing (20 mm), in the south. A full cycle of nursery rearing takes about 6–8 mo with a mean production rate in all settling tanks of 2,000–3,000 seed.m⁻².

Present Problems

In recent years the nursery rearing has not been as easy as in the earlier years of abalone culture in China. On one hand large quantities of postlarvae after settlement (<20 days) fall off the substrate and die. On the other hand lots of young juveniles die when they are removed from substrate to horizontal rearing—usually within 15 days or so. These problems occur in the north and south, resulting in a requirement for more frequent spawning. Since 1995, in the north, a crossbreeding of local *H. discus hannai* with the same species introduced from Japan has generated improvements in this phase of culture. Unfortunately the improvements were not sustained for any longer than 2–3 y (Wang Q., pers. com. 2001) and for this reason some have believed that the species has degenerated by inbreeding after a long term of artificial culture.

Grow-out, Density, Cycle, Output and Market Size

In the north, a special cage for abalone culture is used with 400–600 seed (at >15 mm) per tier at the start of the grow out cycle. These animals are then scattered into other cages to reduce stocking density four times in the 2–3 y of the culture cycle. When they reach size classes of >55 mm and 75 mm they are sold to domestic and overseas markets respectively. For this phase the rafts used hang 25–30 stacks and a stack of 12 tiers crops 25–30 kg of market size abalone (Wang Q., pers. 2001).

In the south, a plastic basket is used in tank culture for 30–35 individual animals of 20-mm shell length. They are then grown in this basket to market size. Some farms raise 50 individuals in this basket at the start and then scatter them to give 30 individuals when they reach 30-mm shell length. A cycle from 20 mm to >50 mm takes about 6–8 mo. At present the small abalone are consumed at 90 individuals per kg (about 47 mm shell length) in the southern markets. A basket crops 400 g in this 6–8 mo. Per cubic

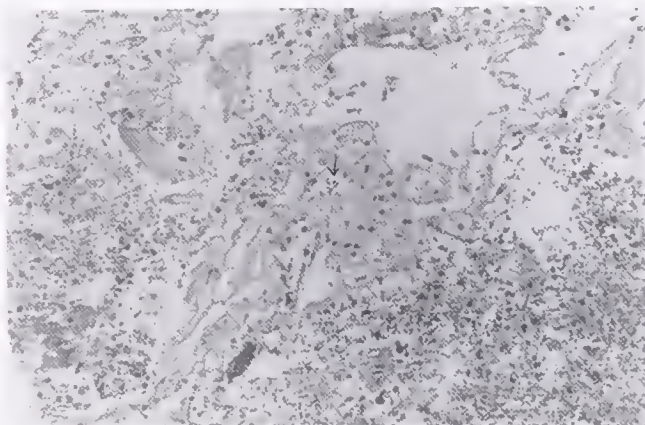


Figure 1. Micrograph of the necrosis area of tissue in the digestive gland of *H. discus discus* presenting with bacterial pathogen. (×200)

meter of water capacity there are 24–30 baskets, cropping 9–12 kg. Mean survival is about 75% (Nie & Wang 2000, Nie & Wang 2001).

The concrete cases used in the south for intertidal culture raise 500–700 juveniles at 10–20 mm shell length and harvest 6–7.5 kg in around 9–12 mo. The survival rate is about 50% to 60% (Qiu C.W., pers. comm. 2001).

Food

China is a country in which abundant culture, of algae such as *Laminaria japonica* and *Undaria pinnatifida* occurs. Natural resources of *Gracilaria tenuistipitata* abound in the south. The brown and red algae are favorites of *H. discus hannai* and *H. diversicolor aquatilis* respectively. The feeding habit of the latter is wide ranging. Its growth rate when fed on dry or salted *Laminaria* is faster than when fed on the fresh *Gracilaria* (Nie et al. 2000). Additionally *Encheima gelatinae*, raised in Hainan, is also supplied for abalone food.

Artificial feeds for the nursery stage are very popular in China. There are powders and different sizes of granules. A few farms have fed on flat feed for the nursery stage. The growth rates of abalone, fed on artificial diets, are faster than the growth rates of those fed on algae (Nie et al. 1986, Nie et al. 2000).

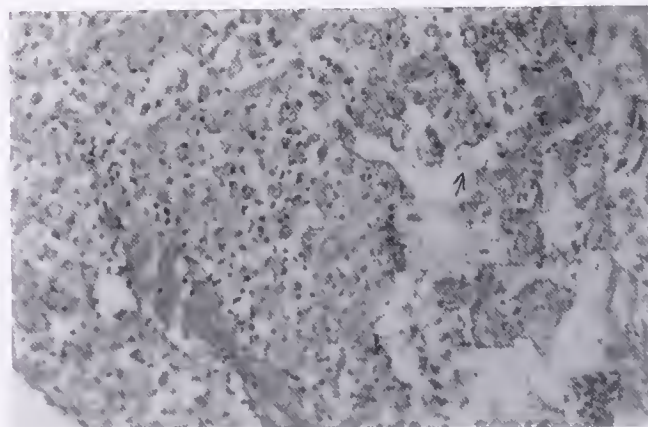


Figure 2. Micrograph of partial necrosis of the tissue in digestive gland of *H. discus hannai* where infiltration by inflammatory cells and many bacilli could be observed. (×400)



Figure 3. Micrograph of the oedematous cells, degenerated mitochondrion and dilatation of endoplasmic reticulum in the digestive gland of *H. discus discus* infected by *Vibrio*. ($\times 4,800$)

Disease

Main Diseases and Enemies

Bacteria: Bacterial disease, especially *Vibrio* is common in aquaculture. An epidemic disease of abalone occurred first in Dalian, northern China in 1993. It is called "pustule disease". The pathogens are *V. fluvialis* and *V. campbellii* (Nie et al. 1995a, Ma et al. 1996). The outbreak that summer caused the loss of 60 tonnes of adult abalone ($\sim 5\text{cm}$ – 8cm). Following that event the epidemic diseases caused by *V. vulnificus*, *V. alginolyticus*, and *V. metchnikovii* were successively identified in Fujian and Guangdong (Nie & Wang 2000) (Figs. 1, 2, 3). Additionally a *Flexibacter* spp. disease has occurred in Guangdong (unpublished data). All are capable of causing high mortality in some situations.

Virus: So far the most serious disease caused by a virus epidemic occurred in the spring of 1999 in Dongshan County, the south of Fujian. It caused 100% mortality at 22 farms within 43 days. A globular virus of dimension $5\text{--}80 \times 120\text{--}150\text{ nm}$ was identified (Huang et al. 1999) (Fig. 4). In the early winter from 1999 to 2002, at about 21°C water temperature, it reappeared at Dongshan and spread to Guangdong. Many farms were infected.

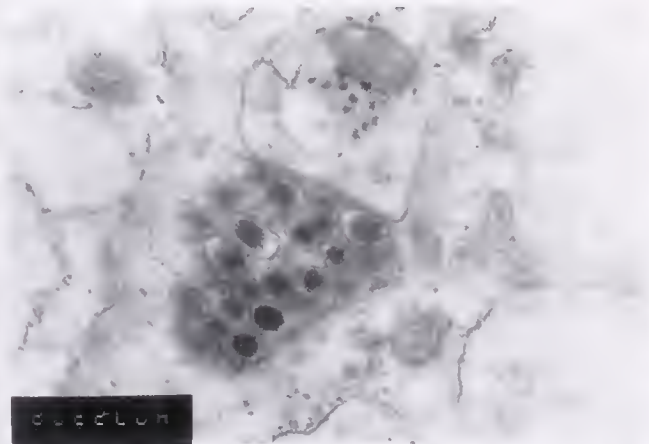


Figure 4. Micrograph of the bladder coat of the virus in the digestive gland of *H. diversicolor aquatilis*. ($\times 40,000$) (courtesy Y.Y. Huang et al. 1999)



Figure 5. Abalone, *H. diversicolor aquatilis*, secreted nacre around its conical appendage due to the settlement of *Polydora*.

Those farms that were infected could not escape another outbreak the next winter (unpublished data). It is clearly a terrible disease for abalone. The clinical signs include; tank water turbid and bubbly because of vomit; after death an atrophied and dark foot muscle still settles on the wall (or basket) of the tank (Huang et al. 1999).

Polychaeta: *Polydora* spp. occurs in the shell of abalone at some farms in the south. They live on the surface of the inside of the shell or in a cave in the rear ventral edge of a shell. The abalone secretes nacre in an attempt to form shell to resist the worms harassment (Fig. 5). The infected abalone becomes very lean and death finally occurs. The smallest infected abalone found was 17-mm shell length *H. diversicolor aquatilis*. This animal had a cave of the worm, 12-mm in length (Nie & Wang 2000). Another clinical sign associated with *Polydora* is rupturing of the mantle at the point of the visceral mass (unpublished data) (Fig. 6). This is called "ruptured abdominal disease." An infection of *Polydora* also caused high mortality at some farms. It is possible that the high mortality occurred as a result of an infection with *Vibrio* synchronously.

In addition to the above diseases, notable but occasional attacks of parasitic protozoans such as *Sporozoa* (unpublished data) (Figs.



Figure 6. Rupture of the mantle (↑↑) at the point of the visceral mass due to the settlement of *Polydora*, and borings of *Polydora* (↑).

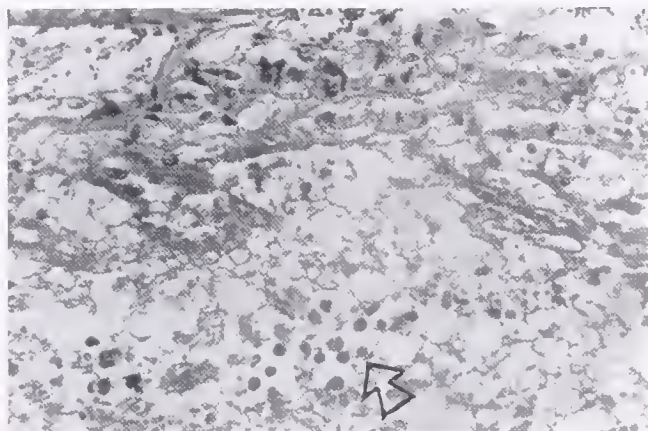


Figure 7. Micrograph of tissue of the digestive gland of *H. diversicolor aquatilis* parasitized by ameboid corpuscles. ($\times 400$)

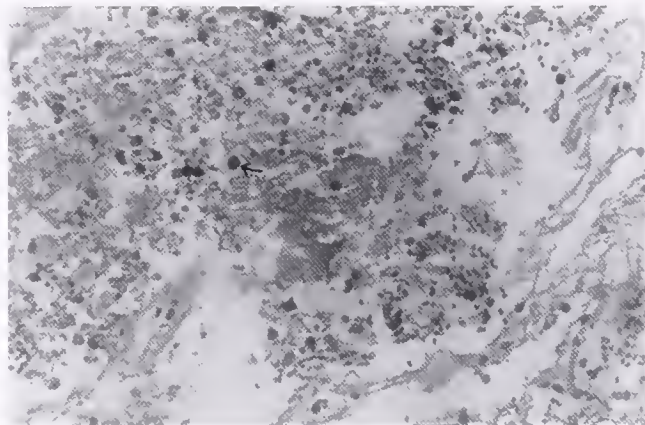


Figure 8. Micrograph of tissue of the digestive gland presenting diffuse necrosis and merger infection by bacteroid pathogen and ameboid corpuscles. ($\times 400$)

7, 8), *ciliata* (Li et al. 1999) have occurred. Another virus disease called "crack shell" caused by a globular virus of 90–140 nm (Li et al. 1998) occurs occasionally.

Cure and Prevention

Some antibiotics such as chloramphenicol, furazolidone, ciprofloxacin and cotrimoxazole, and similar are effective for inhibition of bacteria in seawater. Among these the first 2 are the most effective, however, they are no longer allowed in China. They do not usually cause antimicrobial resistance, and they are both cheap (Nie & Wang 2000). In tank culture the animals are soaked and aerated continually in a 3–4 ppm solution of an equal mix of chloramphenicol and furazolidone for 4–6 h a day. A course of cure takes 3–5 days (Nie et al. 1995a, Nie & Wang 2000). In cases of very high mortality it can be treated twice a day (unpublished data). In most cases the deaths can be controlled. In addition to increasing the frequency of use of the drug, the dose can also be gradually increased. Some effects are obtained by combination of the above antibiotics with a bactericide, such as chlorine dioxide (ClO_2), sodium dichloroisocyanurate, and bromochlorodimethylhydanto all soaked simultaneously with the antibiotic (Nie & Wang 2000). To this date there is no way known to treat the *Polydora* and virus diseases.

Consumers and Pricing

Most products are sold live in domestic markets. A few are consumed frozen and as dry food. The people of Guangdong are the highest consumers. Recently abalone are not only sold to restaurants but also to families. Large cities near the coasts have become the second largest consumers. Some *H. discus hannai* is exported to Japan and South Korea. Some live and canned abalone is also imported from Australia and South Africa. The price of small abalone has declined to US\$ ~10–25 kg^{-1} whereas *H. discus hannai* and *H. discus discus* are selling at around US\$ ~36–46 kg^{-1} .

Prospects

The development of abalone culture is expected to be slow in the future, with output remaining steady. In the north, the extensive cultivation by releasing seed on the sea bed will be increased under the "Law of Use and Administration of Sea Areas." Producers will be expected to pay more attention to the prevention and cure of disease and to breeding technology for improvement of culture quality and disease resistance.

ACKNOWLEDGMENTS

The authors thank Dr. Steve Edwards for rewriting the paper and his helpful criticism.

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ABALONE MARICULTURE IN CHINA

GUOFAN ZHANG,¹* HUAYONG QUE,¹ XIAO LIU,¹ AND HUASEN XU²

¹Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, People's Republic of China;

²Zhanjiang Marine and Fishery Bureau, Zhanjiang 524074, People's Republic of China

ABSTRACT Remarkable progress has been made in recent years in abalone cultivation in China. Beginning in the early 1980s, commercial scale mariculture of abalone has involved 2 species: Pacific abalone, *Haliotis discus hannai* Ino and the small tropical abalone, *H. diversicolor* Reeve. Intensive culture of *H. d. hannai* is distributed mainly in the northern China region, Liaoning and Shandong Provinces with an annual production increasing from about 47 mt (1992) to 4500 mt (2003). The small tropical abalone is the dominant species with a total production of *H. diversicolor* reaching 3878 mt produced in 2001 in Fujian, Guangdong and Hainan Provinces. Seeds for cultivation are exclusively produced from hatcheries with well-developed technology. Cultivation practices may vary widely among different sea types. Three major forms of grow-out are used in abalone cultivation, land-based tanks, suspended longline cages and intertidal ponds. Abnormal mortality has threatened sustainable development of the abalone industry from its inception. During 1992 to 1996, many facilities in the north ran at a loss due to mass mortality occurring in juveniles and seeds. Mortality usually occurred in the postsettlement nursery phase when spat remain on settlement plates, characterized by cease of dietary intake and falling from the substratum. The cause of this large-scale mortality remains as yet unknown. The same situation occurred in Fujian from 1996, after 2 y of large-scale farming, and has already spread to all other provinces of abalone mariculture. Genetic improvement of abalone has drawn much attention. Hybridization between different populations from Japan and China has been achieved to obtain significant heterosis with growth and survival. Preliminary results on *H. d. hannai* have shown that survival of selective strains is significantly higher than normal controls.

KEY WORDS: Abalone, *Haliotis discus hannai* Ino, *H. diversicolor* Reeve, mariculture, seed production, grow-out, disease, abnormal mortality, hybridization, heterosis, selection breeding, marketing, China

INTRODUCTION

Six species of abalones are naturally distributed along the coast of China, *Haliotis discus hannai* Ino, *H. diversicolor* Reeve, *H. asinina* Linnaeus, *H. ovina* Gmelin, *H. planata* Sowerby, *H. varia* Linnaeus, and *H. clathrata* Reeve (Nie 1992). Abalone aquaculture has been dominated by *H. discus hannai* and *H. diversicolor*, whereas culture of *H. asinina* has drawn considerable attention in recent years.

Experimental production of abalone seed in Pacific abalone was conducted in 1977 (Chen et al. 1977) in China, and commercial seed production was accomplished in 1987 in Dalian, Liaoning. From 1987 to 1992, abalone aquaculture in China grew steadily with most research focused on the development of hatchery seed production techniques and grow-out modes. A series of key techniques involving spawning, larval rearing, and juvenile and seed nursing were established. Grow-out systems including culture in suspended longline cages, land-based tanks, intertidal ponds, and tanks were also established.

From 1992 to 1996, however, abalone aquaculture suffered severely from disease in the northern provinces of China. Abnormal mortality of cultured abalone seed, *H. d. hannai*, was first observed in 1992. The mortality problem became evident after 1994, when most abalone hatcheries suffered severe loss of abalone seed, either larvae or juveniles. From 80% to 90% mortality has been reported. During this period, approximately 20% to 30% of 1–3-y-old cultured abalone suffered abnormal death. Meanwhile, wild abalone stocks also suffered mass mortality. Therefore, output of abalone in northern China decreased considerably during 1996 to 1998.

From 1997 aquaculture of *H. d. hannai* in northern China rejuvenated and entered a stage of rapid and stable development.

Success of abalone industry, in north China, resulted from improvement of seed production techniques and implementation of sanitary measures.

During the 1990s, commercial cultivation of the southern abalone, *H. diversicolor* emerged and then expanded rapidly in Southern China. The first trial of hatchery seed production was conducted in Shenzhen in 1992, following the procedure for *H. discus hannai* in the north. This trial, however, came to an unsuccessful end. A package of cultivation techniques was well developed in Taiwan province and popularized in southern provinces of China from 1994. Subsequently, seed production of *H. diversicolor* on a commercial scale expanded until 1998 and saw the rapid development of abalone farming in southern China. Mass mortality occurred initially in January 1999 in Dongshan, Fujian, and then in Shantou, Guangdong. Some companies lost all their abalone. The problem has now spread to Hainan and Guangxi. Most of the abalone hatcheries on the southern Chinese coast are now running limited operations or considering closing.

To summarize, intensive culture of *H. discus hannai* is mainly distributed in north China, with the majority of the production in Liaoning and Shandong. *H. diversicolor* is the dominant cultured species in south China. Abalone culture has become one of the most important components of molluscan mariculture in China. Abalone holds the top position in terms of commercial value among farmed molluscan products. Mariculture production of abalone increased steadily during the 1990s. Despite production of *H. diversicolor* undergoing a decline since 2000, total output of marketable abalone has continued to grow from 1997 onward. This growth is mainly attributable to the rapid increase in production volume of *H. d. hannai*.

In this study, development of large-scale cultivation of abalone, in association with technology, as well as marketing of abalone is reviewed. Meanwhile, problems arising from abalone mariculture and possible countermeasures are discussed herein.

*Corresponding author. E-mail: gzfzhang@ms.qdio.ac.cn

SEED HATCHERY PRODUCTION

Sufficient seed supply is crucial to the development of abalone mariculture. All abalone seeds are hatchery produced with sophisticated technology. For *H. discus hannai*, seed production typically begins in early spring with broodstock conditioning at gradually elevated temperatures and stabilization at 20°C. The most widely used and effective method for abalone to ripen is by effective accumulative temperature (EAT) control. Experiments with Pacific abalone demonstrate that the required EAT is 800°C-day for males and 1000°C-day for females. Meanwhile, plenty of algal food, *Laminaria japonica*, *Undaria pinnatifida*, and *Ulva pertusa* should be provided with this conditioning method. However, conditioning at elevated temperatures is not effective for *H. diversicolor*. Alternately, conditioning is conducted for tropical abalone by lowering culture density to 10–15 individual/cage and adding supplements of fresh macroalgae.

To induce spawning, well-developed abalones are exposed to air for desiccation and then subjected to ultra-violet-irradiated seawater. Adult spawning is usually induced by combined thermal shock and UV-treated seawater exposure. Few hatcheries use hydrogen peroxide to induce spawning for *H. diversicolor*. Males usually are induced 1 h later than females because the former are more sensitive to inducement than the later. Fertilized eggs are incubated at 21°C to 22°C and 22°C to 30°C for the northern and southern types, respectively. Approximately 60 h postfertilization, *H. d. hannai* larvae are ready for settlement. Eyed larvae are set on the collecting plates made of transparent corrugated plastic with diatom-precoated for *H. d. hannai*. In southern China, plastic films (usually polythene with 0.2–0.3 mm thickness), which are precoated with diatoms, are widely used for *H. diversicolor* larval collection. Using plastic film has achieved satisfactory settlement percentage and postsettlement survival. However, this stage is critical and survival after settlement is the biggest challenge for abalone seed production. The diatoms must be supplied in sufficient quantities as well as in good quality to ensure the growth and survival of postlarvae. Water quality and light level should be carefully controlled during this critical period.

Juveniles are manually moved from settlement plates to nursery culture plates at approximate size of 3–7 mm. For *H. discus hannai*, spat are usually transferred to large punctured plastic plates with dark color. For *H. diversicolor*, however, spat are reared in concrete nursery tanks. Square cement plates, with feet on the four corners, are placed on the bottom of the tanks. During early juvenile stages, an artificial diet is exclusively used as food supply. Several commercial diets, from various diet producers, are available to aquaculturists. The major ingredient of artificial diets is dried kelp powder and fish meat powder. Fresh kelp is the principal food for older juveniles. The quality of artificial diet plays an important role in postsettlement survival.

GROW-OUT SYSTEMS

There are at least 3 major grow-out methods being used widely for abalone culture. First, grow-out in land-based tanks is a popular system, and the most intensive and sophisticated. The method is applicable to both the cultured species. In southern China, this method had developed in Taiwan before 1994. It has become the predominant culture method for the abalone culture industries in South China. The major husbandry measures include adequate feeding, control of water flow, periodic elimination of abalone waste, and adjustment of the culture density. Usually this grow-out

system has a high running cost, including cost for water pumping, heating, and aeration. For northern China, this grow-out system is applied less than in southern areas. One of the most successful facilities running this grow-out system is Xinda Products Co. located in Dalian, Liaoning, retaining stable full production for many years.

The second cultivation form is in cages or barrels on suspended longlines. This system was initially tried in north China in the late 1980s. For the southern coast, the first trial was conducted in Shenzhen, Guangdong, in 1992. Relatively fast growth at lower costs could be obtained with this abalone grow-out system. Therefore, this method has been widely adopted along the coast of China. Feeding with macro-algae is usually conducted weekly. The grow-out usually lasts for 8–13 mo for *H. diversicolor* on the south China coast.

The third form is culture in intertidal ponds. The seed of *H. diversicolor* is put into a cement tank, diameter 120 cm and height 60 cm, wall thickness 5 cm, with a cover and a rack for the abalone settlement. There are several poles in the cover, bottom, and wall for the water change between the inner and outer parts of tank; 500 to 700 seeds of 2 cm shell length can be placed in each tank. Tanks are set in the low intertidal or upper subtidal zone and fed with fresh algae once per week or more according to the weather, and the amount is 20% to 30% of the total weight of abalone in the tank. The grow-out of abalone usually takes an average of 7 mo, varying from 5 to 11 mo. This is a closed system and has first applied in Zhanjiang, Guangdong in 1993. From the unprecedented success of this culture mode in 1997, an upsurge in popularity occurred during 1998 to 2000. Since 2002, however, the practice has declined sharply due to abnormal mortality from early seed to adult. One reason is that rapid development of abalone farming has resulted in environmental disruption in coastal water and epidemic pathogens.

In northern China, intertidal farming emerged in 1998 when hybrid seeds were successfully produced and the over-wintering problem solved. Some cement ponds have been built in the low intertidal zone with an area 0.5–1.0 hectare, with stones placed in the pond for the settlement of abalone. Ponds are about 1–2 m(s) depth depending on the position and the system of water exchange. There is a net along the pond wall to prevent abalone escape. No tank was used in the north. The seed size is 2.0–2.5 cm shell length and 15–20 individual/m². The grow-out needs 16–20 mo to reach the size for market in Qingdao. Generally, there is an opening system for abalone grow-out, which has been developing quickly off the coast of Qingdao, Shandong. The ratio of marketable abalone produced from this grow-out system has steadily increased in recent years. In 2003, production of marketable abalone reached 2615 mt in the Qingdao area, accounting for over 58% of the total yearly output in Shandong. Grow-out management includes periodic feeding on macro-algae and removal of dead bodies. One disadvantage of this opening system in the intertidal zone is that it impedes natural beach scenery.

In addition to the above mentioned grow-out modes, abalone can also enhanced on the bottom of the subtidal zone. This has been used in a couple of coastal area, such as the Changhai County in Dalian, Liaoning.

During the grow-out period, abalone are fed primarily on the macroalgae, *Laminaria japonica*, *Undaria pinnatifida* or *Ulva pertusa* for *H. d. hannai* and *Gracilaria* sp. for *H. diversicolor*. Additionally, *Laminaria* and other macroalgae are used partially for *H. diversicolor*. During summer, following the completion of the

kelp harvest, kelp supply could be in short supply for 2–3 mo. Salted or cold-stored *Laminaria* substitutes for fresh kelp as the abalone food supply during supply problems of fresh food. Both salted and cold-stored *Laminaria* should be processed before use.

MARKETING OF MARICULTURE PRODUCTS

Aquaculture products of abalone are primarily sold fresh in domestic markets of China. Tinned products are available at some abalone farms. Most canned products were sold in the overseas market.

CHALLENGES TO ABALONE MARICULTURE

Mortality frequently occurs during the first month of the juvenile phase, following the movement of juveniles from settlement plates to the nursery culture plates, especially when the juveniles are taken off the diatom plates at <5 mm shell length. It is generally believed that the change of feeding habits is the major cause of this high mortality. Survival in this critical nursery period has dropped to lower than 5% at the lowest level during the early 1990s. Survival of early juveniles has been much improved by hybridization breeding, declining juvenile density in the collecting plate, and extension of diatom feeding, therefore, allowing for removing the juveniles, at a larger size than normal, to the nursery culture plates (Zhao & Zhang 2000). In particular, hybridization was made between Japan and China's broodstock to produce hybrid seeds. When all diatoms on the settlement plate are consumed, juvenile abalone (approximately 2–3 mm) are transferred to another batch of transparent corrugated plastic plates on which diatoms are well covered. Therefore, size of juvenile abalone reached 5–7 mm when transferred to the nursery culture plate, enhancing the adaptive ability of juveniles to artificial diets. The practice of transferring juvenile abalone is now becoming part of routine hatchery procedure. With the application of hybridization and plate-transfer techniques, production of *H. d. hannai* remains stable in the northern Chinese coast areas.

This problem, however, was not solved completely due to incomplete understanding of the physiologic variation associated with the change of feeding habits, following larval metamorphosis. In addition, a lack of quality control, with respect to broodstock, often leads to failure of utilization of heterosis. Hybrid abalone seeds have now been widely adopted throughout the northern coasts. Nevertheless, breeding of high quality lines of abalone will be the best solution in terms of genetic modification.

Availability of adequate commercially formulated diets is another problem for the abalone aquaculture industry in China. Abalone is a slow feeder, therefore, reducing leaching of water-soluble ingredients from artificial diets would be key to good diets. Furthermore, dietary essentials should be balanced to meet the nutrient requirement of abalone. As yet, however, nutritional requirements of the cultured species have not been fully demonstrated.

RECENT ADVANCE AND PROSPECTS

With the development of abalone aquaculture, the demand for abalone lines that are genetically improved has become a common concern. To meet this urgent demand, the Ministry of Science and Technology of China has provided funding for genetic improvement and breeding of mass culture species (e.g., *H. d. hannai*).

Heterosis has been used extensively in Pacific abalone farming. Most seeds used for mariculture are hybrids of the Japanese and Chinese populations, all showing a significant heterosis in growth and survival with 17.98% in shell length, width 22.07%, total weight 61.93%, and survival 180% compared with the control. Heterosis is the foundation of a modern abalone breeding program. The selective strain study has made significant progress with a selective line obtained. This abalone has the characteristics of uniform size, fast-growth, and high survival in all developmental phases. The selection pressure in the first generation was conducted at 0.2%. Selection was undertaken for 2 successive generations and F_3 offspring have been obtained. This abalone line is a high-quality candidate for aquaculture and has the potential for promotion of the abalone culture industry in China.

To facilitate abalone breeding, marker assistance selection (MAS) based on molecular markers has been conducted in cultured abalone species. For example, the random amplified polymorphic DNA (RAPD) technique has been used to investigate genetic differences of the abalone, *H. d. hannai* in 4 progeny families from different geographical populations in China and Japan, as well as genetic variation between parent abalone (from Japan and China, respectively) and their hybrid offspring (Wan et al. 2001, Zhang et al. 2002). The construction of full length cDNA library of *H. d. hannai* from F_1 offspring of J_1R_h family had been fulfilled (Liu et al. 2004) and EST sequencing of the "Chinese Red" line of abalone (Liu et al. 2003) and genetic linkage mapping for high quality family in growth and survival are ongoing.

Chromosome manipulation has been conducted in both *H. d. hannai* (Wang & Zhang 1990) and *H. diversicolor* (Rong & Weng 1990). Triploids were induced with various methods including cold shock, treatment of cytochalasin B, or of 6-dimethylaminopurine (6-DAMP) in the abalone, *H. d. hannai* (Sun et al. 1993, Zhang et al. 1998). Unfortunately, no successful application of triploid abalone in mariculture has been reported until now.

High mortality occurring during abalone culture, either nursery or grow-out stage is another crucial issue for both abalone aquaculturists and researchers. Epidemiology investigation and pathogen identification are ongoing and progress has been made. Two major diseases have been reported in China. First, a fester disease has been reported in juvenile abalone. The pathogenic bacteria of the fester disease was verified as *Pseudomonas fluoresce* and some antibiotics, including Kanamycin, Oxolinic, and Furaxone have been found to be effective in curing this disease (Ye et al. 1997). An Elisa method for detecting the pathogenic bacteria of the fester disease in cultured juvenile abalone was reported (Ye et al. 1998). Second, a serious pustule disease was also found, usually in grown-out abalone, caused by *Vibrio fluvialis* (Liu et al. 1995). Various antifibrosis vaccines for cultured abalone *H. d. hannai* were examined. There is still a long way to go before mortality can be effectively controlled. The occurrence of inexplicable mortality will complicate abalone aquaculture for the near future.

ACKNOWLEDGMENT

The authors thank Dr. Huang Bo, Dr. Ke Caihuang, and Miss Lin Zhishu for providing valuable first-hand information on abalone mariculture of various provinces; and Miss Kirsty for her review in English. This study was financially supported by National High Tech R & D Plan (863 Plan), grant number 2001AA621070 and 2003AA603023.

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STATUS OF THE ORMER (*HALIOTIS TUBERCULATA* L.) INDUSTRY IN EUROPE

S. M. H. HUCHETTE^{1,2,*} AND J. CLAVIER¹

¹LEMAR Laboratory, European Institute for Marine Studies, University of Western Brittany, France;

²Zoology Department, University of Melbourne, Victoria, 3052, Australia

ABSTRACT The ormer (*Haliotis tuberculata*) occurs from the Senegal coast in the south to the Cotentin peninsula of France in the north. Commercial fisheries currently operate in France and Spain. The French abalone fishery was opened in 1994. Abalone catches are regulated by a permit and quota system. Total Allowable Catch (TAC) was determined by two initial stock assessments carried out locally in the vicinity of St Malo, Central-North Brittany, and Morlaix, North-West Brittany, in the 1980s. Quotas have since been revised, according to decisions of local fishing committees, without information on the stock situation or independent assessments since the opening of the fishery. In 1996 to 1997, a severe bacterial disease devastated the stock of the main fishing grounds in St Malo and many other places around Brittany. Mass mortalities were correlated with high sea surface temperatures. The stocks have only partially recovered since then, and the total catch has been significantly reduced from about 80 tons to 60 tons per year, resulting in catches significantly lower than the TAC currently implemented. There are no reliable catch per unit effort data available. The absence of a strong enforcement policy, evidence of poaching, and strong activity of the local black market constitute a situation where there is a significant threat to the stocks and sustainability of the fishery. In Spain, the fishery was reopened in 2002, after 10 y of inactivity related to PSP toxins found during imports into the Japanese market. It was closed again, soon after reopening in 2002, because of a major oil spill on the Northern coasts. An abalone aquaculture industry is developing around Europe, mainly in Ireland where *H. discus hannai* and *H. tuberculata* have been imported and acclimatized. There are prospective aquaculture developments in France, England, Spain, and Scotland.

KEY WORDS: ormer fishery, France, *Haliotis tuberculata*, management, *Vibrio carchariae*.

INTRODUCTION

Considering the status of the abalone industry worldwide, with the high commercial value of the product and the collapse of many fisheries, it is likely that the remaining abalone stocks will be put under greater pressure (Gordon & Cook 2001). The reasons for stock collapses are varied, but they may be linked to over exploitation of the resources, unwise management, inadequate enforcement policies, or disease.

The ormer, *Haliotis tuberculata*, is the only European species that reaches a large enough size to be harvested. It occurs from the Senegal coast, in the south, to the Channel Islands in the north (Gaillard 1958, Mgya 1995). It is traditionally fished in western France where it is considered a delicacy.

It was considered as a potential candidate for aquaculture development in the 1970s in France, where technical feasibility studies were conducted (Koike 1978, Flassch & Aveline 1984). At that time, however, these studies concluded that ormer culture was not economically viable due to its low market value (3–10 €/kg) and slow growth. Commercial fishing and mariculture interest of the ormer significantly increased all through Europe in the early 1990s following easier international commerce (rapid transport and globalization of markets) and an increase in its export values to Asia.

In this study, fisheries information was collected by direct investigation in the field, by interviewing professional fishermen and scientists, and from a review of the existing literature. We describe recent changes in the European industry and its current status, analyze management strategies, and discuss potential future developments.

DESCRIPTION OF THE INDUSTRY

Recreational Fishing (France)

The ormer has been fished in France for many years. Fishing restrictions have been established by regional fishing committees,

and are enforced by the Directorate of Maritime Affairs. Restrictions include a minimum shell length of 8 cm (until 1996 then 9 cm), a bag limit of 20, and a seasonal closure from June 15 to August 31. Recreational fishing can only be carried out on foot and the use of a fishing hook is allowed. No breathing apparatus, and no swimming is permitted, and the fisher's head must remain above water at all times. Fishing is carried out only during extreme low tides and is only allowed between sunrise and sunset. Fishing licenses are not required.

The major problem, however, is that few people are aware of these rules because they are not widely advertised on the assumption that "no one is supposed to ignore the law." Recreational fishing is not estimated, however there were 4,000 shore gatherers in the Rade of Brest in 1963 during the equinox tide, 2,000 in 1984 and 1,000–2,000 in 1996 (IFREMER statistics). Clavier (1992) estimated the total catch of these shore gatherers at about 20 tons each year.

Commercial Fishing (France)

Commercial fishing started in 1994 for a preliminary experimental period of 1 y. License holders are SCUBA divers, restricted to only 2 SCUBA tanks per day. Health and safety regulations for professional diving require three qualified divers (commercial diving certificates are required) on board the boat. Abalone, minimum shell length of 9 cm, can be collected with an abalone iron or hook. All abalone fished must be marked before landing with individual plastic seals (colored according to the fishing zone and marked with a personal seal). Five thousand individual plastic seals are given to the fishermen at the start of the season for each ton of quota. Management is funded by the cost of the annual commercial fishing licenses (€152.45/boat per year). A seasonal closure from June 15 to August 31 applies. The control and enforcement of these regulations are carried out upon arrival in the port. If, during a control operation:

- more than 20% of the fish caught are under the minimum legal size, the fishing license is cancelled for 1 y.

*Corresponding author. E-mail: sylvain.huchette@wanadoo.fr

- there are no seals on ormers during transport, the license is permanently removed.

Poaching seems wide-spread. It is likely that well-organized parallel networks supply local markets, Parisian restaurants, and illegal exportations. Prosecution of poachers is slow and less dissuasive than overseas: for example, 2 poachers were sentenced in 1999 to 6 mo in jail and fined €8000 for poaching 28 tons between 93 and 95 (Ouest-France, daily newspaper, September 1999).

There are 5 fishing zones in Northern Brittany and Normandy (Fig. 1 & Table 1). The French management system for abalone fisheries is presented in Figure 2 and is mainly carried out at the local level by Local Fishing Committees (LFC). Each local fishing committee has its own management strategy, with the Normandy one being more distinct than those in the four Brittany zones, because Normandy belongs to a different administrative region. In Normandy, the SMEL (Syndicat Mixte Pour l'Exploitation du Littoral, Blainville, Cotentin), a local research and development center funded by the French Département of La Manche, works closely with the fishermen and IFREMER and provides management advice. In Brittany, IFREMER (Institut Français pour la Recherche et l'Exploitation de la Mer), is in charge of advising LFCs, but it takes a limited part in the decision process. There are no regular stock assessments carried out. As a result, the fishery is in an awkward situation where the quantities of abalone officially fished are well below the Total Allowable Catch in all regions except Normandy (Fig. 3). More recently, following demands by commercial sea-urchin divers, a stock assessment has been carried out by IFREMER in 2002 for the Southern Brittany area and a potential fishing biomass of 18 T has been estimated, and a small fishery may be opened in 2003 (Billy & Péronnet 2003).

A mass mortality in 1996 to 1997 have devastated the stocks in the Bay of Morbihan (Southern Brittany) and in central Northern Brittany (Zone of St Malo). No pathogenic agent have been identified with certainty in the wild, however, Nicolas et al. (2002) isolated and described a bacterial species, *Vibrio carchariae*, as the main pathogenic agent involved in mass mortalities that occurred

at the same time in land based operations. It is genetically and phenotypically close to *Vibrio fluvialis* H (Li et al. 1998) found in China on *H. discus hanai*. Recreational fishing was closed in 1998 and the opening of the commercial fishing season was delayed. Fishing started again in 1999, but catches have remained low in these zones. Divers report that outbreaks of the disease in these areas are still occurring each year late in the summer: severe mortality through all size classes was observed at the opening of the fishing season in September (Jehanno, pers. comm.). Although there is no scientific evidence to support this, the disease possibly becomes dormant during winter and is activated by high summer temperature. Figure 4 presents an interesting correlation between the areas of severe mortality observed by divers and local communities and sea surface temperature at the end of August. The disease is currently being studied more extensively.

Guernsey and Jersey (Channel Islands)

After 8 y of fishing with SCUBA, the commercial abalone fishery was closed in Guernsey in 1973 and has never been reopened since. Only shore gathering during spring tide, outside the reproductive season, is permitted nowadays (Mgaya & Mercer 1994). Commercial fishing using SCUBA, however, may be permitted again because the stocks seem to be recovering (Bossy, pers. comm.).

In Jersey, there is no commercial fishery. Only a recreational fishery is allowed and is conducted by fishing at low tide or "pêche a pied" as it is known locally. Ormer fishing is a very emotive subject in Jersey and is considered part of their heritage by the local community. Abalone aquaculture was attempted recently, but the summer mass mortalities wiped out the entire stock, and thus, there is no large scale aquaculture. After the disease outbreak in 1997, there was a total ban on ormer fishing until 2002. Recreational fishing regulations are complex and include a closed season between the May 1 and August 31, a minimum shell length of 8-cm, and the activity may only be carried out on the first day of each new moon or full moon and the three following days. Breathing apparatus, face visors, goggles, or masks are forbidden while fishing any shellfish other than crab. An abalone population survey carried out in 2002 provided evidence that the population was approaching pre-mortality levels and the ban on fishing is to be lifted (Morel, pers. comm.).

Spain

A commercial abalone fishery operated in Galicia from 1989 to 1993. In 1993, the fishery was closed following the discovery of PSP toxins by the Japanese in imported Spanish abalone. (Bravo et al. 1999, Bravo et al. 2001, Nagashima et al. 1995). There was no local demand to maintain the fishery. Following the study by Bravo et al. (2001) suggesting that toxins were contained only in the epithelium of the side of the foot, the fishery was reopened in April to May 2002. Almost all catches are exported, mostly to Asia. Abalone were sold in their shell at a minimum price of 24 €/kg in 2002. The demand has been variable possibly because of the small quantities offered on the market by the fishermen.

Fishing is carried out over the entire Galician coast, but most of the catches are in sheltered areas, which are more accessible (Juan Freire, pers. comm.). Catches are more important in the south, where large coastal embayments dominate the coast. The more exposed northern coast receives less attention. For example, in Cangas, where a large proportion of the fishery is carried out,



Figure 1. Fishing zones of the French ormer fishery since 1994. Each zone is managed by a local fishing committee.

TABLE 1.

Change in quota tonnage and numbers of divers (in brackets) over time in each fishing zone since the opening of the fishery in 1994.

Year of Activity	Nord-Finistère	Paimpol	S ^t Brienc	S ^t Malo	Cherbourg	TAC (tons)
End 94	9.6 (16)	3 (12)	—	12 (12)	21 (6)	45.6
Early 95	9.6 (16)	4 (12)	—	18 (12)	21 (6)	52.6
95–96	10.8 (9)	6 (12)	—	36 (12)	21 (6)	73.8
97–2000	15 (10)	18 (11)	8 (8)	36 (12)	21 (6)	96
2001–02	10 (10)	18 (11)	8 (8)	36 (12)	21 (6)	91

abalone divers operate from May to September (4 mo). A minimum shell length of 8 cm and daily maximum catch quota of 5 kg/diver with a maximum of 15 kg/boat, are applied. In 2002, Cangas had seven boats with three divers each. Divers often combine abalone and sea urchin fishing. There is no regular stock assessment of the fishery and no information on the extent of poaching. Since late 2002 all coastal fisheries were stopped, following the major oil spill caused by the ship "Le Prestige" in Northern Spain.

North Africa

Although ormers are found on the north-west African coast, very little is known about the fishery. Exploitation of the resources by local fishermen has been encouraged by foreign traders, mostly from Asia. The extent of exploitation of wild populations and any management is not known.

Aquaculture

The first technical studies of ormer culture in France were carried out in the late 1970s and early 1980s by Flassch and Aveline (1984), Koike (1978), Koike et al. (1979), and Cochard (1980). They developed techniques for rearing, conditioning, and spawning (reviewed by Mgaya & Mercer, 1994). These studies concluded, however, that ormer culture was not economically viable, mainly because of the low market value of abalone at the time. Ormer culture has, therefore, not developed much in Europe. Ireland and the Channel Islands (2–3 tons/year) are the only established producing countries today. Ireland has introduced and

acclimatized both *H. tuberculata* and *H. discus hannai*. Few studies have been carried out so far to compare the performance of the two species. Cultivation attempts have also been carried out in Scotland (Kelly & Owen 2002).

In France, culture techniques have been perfected more recently by the SMEL. The duty of the SMEL is to provide technical support to any company willing to establish a farm in the Département of La Manche. They contributed to the first study of the bacterial epidemic because they lost a great part of their stock during the first outbreak (Nicolas et al. 2002). Their recent work also included preparation of broodstock for reproduction (inversion of photoperiod cycle for spawning during daytime and reduction of the conditioning cycle to 1–2 mo), techniques for live transport, settlement induction using GABA, stocking density, and ammonia management in recirculation systems (Richard, pers. comm., Basuyaux & Mathieu 1999). So far, only one commercial hatchery has been established in the department of La Manche in 2002, and it aims to produce small cocktail-size abalone to supply Parisian restaurants. One attempt at grow-out farming in sea cages was also started near Paimpol in Brittany in 2002, but the farm was stopped in its early development phase in 2003 for private reasons (Arlin, pers. comm.).

DISCUSSION

The ormer is a traditional delicacy in France and the channel Islands (Clavier 1992, Mgaya & Mercer 1994). Ormers were fished and consumed some thousands of years ago and are even found in archeological sites around Brittany (Chauvaud, pers. comm.). In spite of this local market and recreational fishing pressure, ormer populations have persisted until now. The European abalone industry has, however, faced major problems in the last decade from the *V. carchariae* disease outbreak, oil spills, and paralytic shellfish poisoning. In addition to these problems, the high commercial value of abalone is constantly increasing pressure

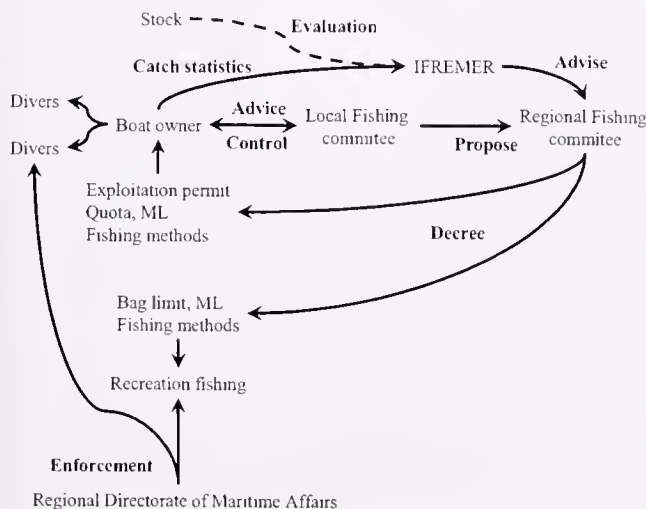


Figure 2. Administrative management of commercial and recreational ormer fisheries in France.

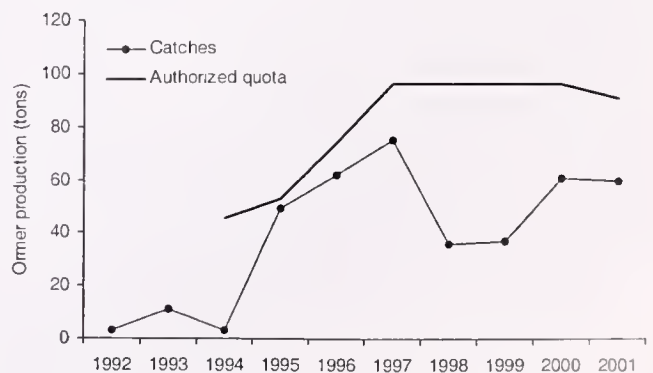


Figure 3. Quotas and official catches (FAO, 2000) over time in France.

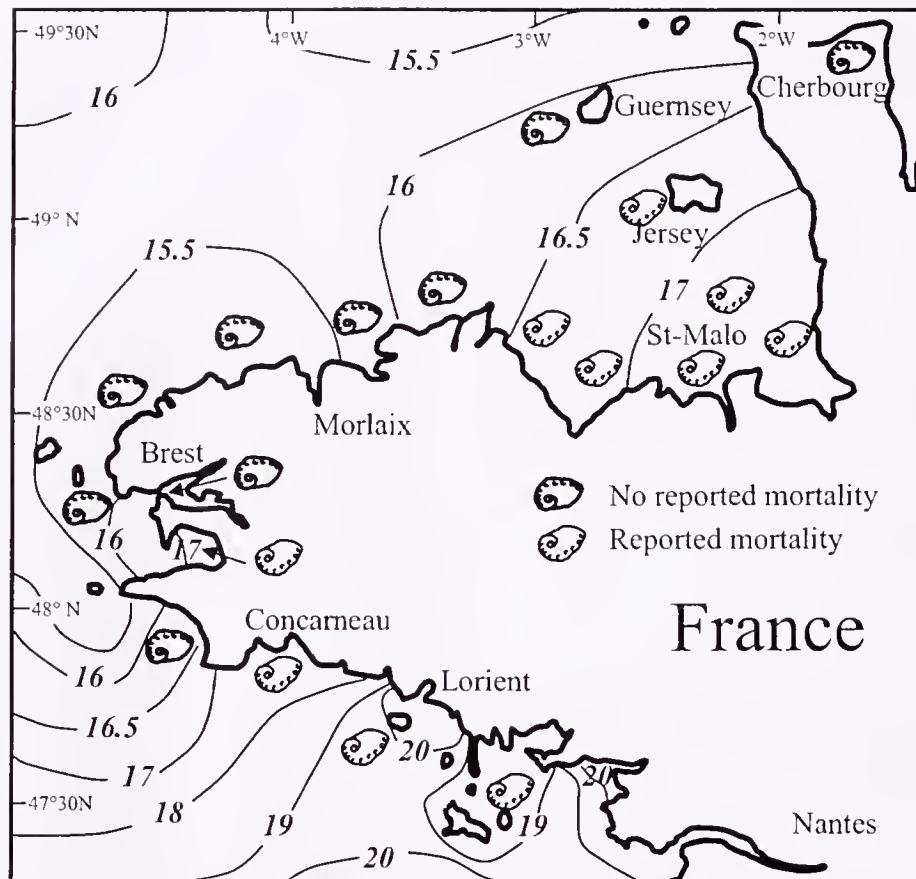


Figure 4. Map of Brittany showing the areas where mass mortalities have been witnessed (Mazurié & Richard 1999, Mazurié et al. 1999, and other testimonies collected by the authors) and the isotherm for the summer maximums (Compiled from: SAF O&SI (Satellite Application Facility) of EUMETSAT/Météo France in Lannion; Vincent & Kure 1963, Lumby 1935).

on resources. Although difficult to assess, poaching is likely to considerably affect wild stocks because they are located in populated areas of Europe. Commercial divers often report that rocks have been turned over by poachers leaving devastated reefs behind them (Billy & Péronnet 2003). Possibly however, the cryptic behavior of ormers (Mgaya & Mercer 1994, Clavier & Richard 1984) may prevent the species from being fished out from the grounds where poaching is high, because larger rocks and deep crevices cannot be harvested.

In France abalone is considered as a minor fishing resource mainly because of its small TAC, low economic significance, and because it can sustain only a small population of fishermen. Management of the fishery is not, therefore, a priority and may seem inadequate. Although quotas were initially decided conservatively, allowing to small and very localized initial stock assessment, there are no fishery-dependent or independent stock assessments regularly carried out. Fishing pressure is, thus, not adjusted according to the biomass present. For example, in the area most affected by the mass mortality in 1996 to 1997, quotas were never reduced and the ban on fishing lasted for only 6 mo.

It is well known that overfished abalone stocks are slow to recover when fishing is stopped, and may collapse if fishing continues (Davis et al. 1996). The ormer stocks of Guernsey took at least 30 y to recover from a few years of intensive fishing. Sustainable management of an abalone fishery requires much attention

and must be tailored to protect the most vulnerable reefs (Shepherd 2000). Although management carried out by local fishing committees in France, they sometimes fail to decide on the closure of threatened fishery grounds (e.g., in the St Malo district where annual catch have become very low after the mass mortalities of 1996 to 1997). Local fishing committees often lack knowledge and expertise about the biology, ecology, and dynamics of abalone populations to take informed and preventive management decisions. Little information is collected about the wild populations, mainly because of a lack of financial support. The quota distribution often means that each fisherman receives too little quota to ensure a living and have to fish crustaceans or echinoderms to complement revenues. Higher revenue from a single activity could possibly increase the concern of fishermen to protect the stocks. In the department of La Manche, where there are less divers and more quota per person, the stocks appear to sustain a healthy industry (Richard, pers. comm.).

Commercial and recreational fishermen and seafood traders usually support the argument that poaching remains common in France, probably because of a lack of significant enforcement of the regulations by local authorities. It is common that occasional fishermen are not informed of the fishing regulations. Enforcement and broader public communication about the regulations and the biology and vulnerability of abalone populations, could be the first steps to prevent decline. Enforcement should be carried out within

an international framework to prevent the development of an organized poaching network (Campbell 2000, Daniels & Floren 1998). Fines and jail sentences should be similar to those imposed overseas (Masland & Barbee 2003).

The sustainability of European ormer fisheries cannot be assessed reliably, because there are too many unknown parameters remaining. The situation is alarming, however, considering the reasons for the collapse of many other abalone fisheries worldwide (Tegner 2000, Hobday et al. 2001, Shepherd et al. 1998, Campbell 2000, Shepherd & Rodda 2001), little has been done to protect European abalone populations. More research is required to understand stock dynamics, the effects of *V. carchariae* on the populations, and the risk of collapse of the population under the current management regimen. European research collaboration might en-

hance the development of the ormer industry and help to prevent the collapse of its stocks.

ACKNOWLEDGMENT

The authors acknowledge the help of Gerard Veron (IFREMER Brest), Olivier Richard & Olivier Basuyaux (SMEL, Blainville), Juan Freire (Universidad de Coruña, Spain), Simon Bossy (Fisheries officer, Guernsey), Greg Morel (Fisheries officer, Jersey), Stephanie Billy (Tethys environment, Lorient), André Arin (oyster farmer, Paimpol), Jehanno (fisherman, St Malo), Laurent Chauvaud (CNRS, Brest), and many others for providing the information used to compile this article. Special thanks to Dr. Rob Day (University of Melbourne, Australia) for his useful suggestions to improve the manuscript.

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GROWTH AND SURVIVAL OF POST-LARVAL ABALONE *HALIOTIS DIVERSICOLOR SUPERTEXTA* (LISCHKE) USING AN ALTERNATIVE CULTURE METHOD IN THE LIGHT AND DARK

ADRIAN E. STOTT,¹ TOSHIO TAKEUCHI^{1*} AND YASUYUKI KOIKE²

¹Laboratory of Fish Culture, Department of Aquatic Biosciences, Tokyo University of Fisheries, 4-5-7 Konan, Minato, Tokyo 108-8477, Japan; ²Banda Marine Laboratory, Tokyo University of Fisheries, Banda, Tateyama, Chiba 227-0036, Japan

ABSTRACT Stott's Abalone Postlarvae Production System (SAPPS) was developed as an alternative method for settling larvae and raising postlarval abalone. It allows continuous hatchery production of abalone without using live food. The new system was tested in a 28-day trial, both in the dark and light, on *Haliotis diversicolor supertexta*. Plates were sprayed with a mixture containing commercial, artificial micro particle diet 1 or 2, and a 1% agar solution and assigned a 10-L flow-through tank, either in the dark or light. The four treatments—Diet 1-dark (D1-D), Diet 1-light (D1-L), Diet 2-dark (D2-D), and Diet 2-light (D2-L) were replicated 3 times. The chemical composition of the two diets was similar although lipids were 21.05% in diet 1 compared with 13.74% in diet 2. Final survival was not significantly different ($P > 0.05$) among the treatments and ranged from 14.8% to 31.4%. The variable results for larval settlement and low postlarval survival in the first week was most likely due to unsatisfactory preconditioning of plastic plates with juvenile abalone trail mucus or weak settlement induction of this mucus. Although the light/dark factor was not significantly different ($P > 0.05$), abalone postlarvae fed diet type 2 (D2-D and D2-L) were significantly larger ($P < 0.05$) than those fed diet type 1 (D1-D and D1-L). The average growth rate per day was $43 \pm 4 \mu\text{m}$, $44 \pm 5 \mu\text{m}$, $51 \pm 4 \mu\text{m}$, and $53 \pm 4 \mu\text{m}$ for D1-D, D1-L, D2-D, and D2-L respectively. Diet 2 may have been more nutritionally complete than diet 1. SAPPS was used successfully in this trial to culture *H. diversicolor supertexta* postlarvae, regardless of light and natural food.

KEY WORDS: SAPPS, larval settlement, post-larval nutrition, micro particle diet, abalone, *Haliotis*

INTRODUCTION

Several problems have been identified and documented in regards to the current method of raising abalone on diatom biofilms. Abalone hatcheries often suffer 90% to 100% mortality by 2 mo postsettlement (Searcy-Bernal et al. 1992). Management of these diatom biofilms is often challenging and Hahn (1989) and Seki (1997) suggest that control of the initial food supply is still one of the most critical problems in hatchery seed production. It is a difficult task to maintain a supply of readily ingestible and digestible food (diatoms) for growing postlarvae (Kawamura et al. 1998). Alternative methods to those utilizing living diatoms, for abalone postlarval production, may be beneficial to industry.

A mass culture system for the green alga *Ulva lens* on plastic plates has been developed to provide a settlement cue for larval abalone and provide a source of nutrition for the postlarvae. However, growth rates of postlarvae fed *U. lens* are significantly lower than favorable diatom species (Kawamura 1996, Seki 1997, Daume et al. 2000). Alternatively, Buchal et al. (1998) reported that postlarval *Haliotis rufescens* could be raised on dulse (*Palmaria mollis*) but direct utilization of dulse as a principle food source was only observed in abalone >3 mm in length.

Olin and McBride (2000) suggested that artificial diets are sometimes used by commercial abalone hatcheries to feed postlarval abalone once they have reached 2 mm in shell length. Stott et al. (2002) reported that young postlarval *Haliotis discus discus* grew well on a micro particle diet that was bound within an alginate gel which had been thinly pasted onto plastic plates. In a follow-up study, Stott et al. (2003a) developed a method for settling larval *H. diversicolor supertexta* directly onto plastic plates coated with the micro particle diet/alginate gel. Growth and survival rate of abalone postlarvae using this method was comparable

to that of post larvae grown using the diatom biofilm method. However, the alginate gel method would not be suitable for large-scale production of postlarval abalone (Stott et al. 2002, Stott et al. 2003b).

Consequently we focused our research on addressing this limitation and SAPPS was developed. This new method utilizes the temperature dependant setting properties of agar to cover plates, via spraying, with a micro particle diet/agar gel mixture. Commercial scale trials were previously conducted to test growth and survival rate of postlarval *H. diversicolor supertexta* using SAPPS compared with that using conditioned diatom biofilm (Stott et al. unpublished data). After 42 days abalone grown using SAPPS had reach an average size of 3.2 mm with a survival rate of 51.7% compared with a size of 2.2 mm with a survival rate of 10.6% for conditioned diatom biofilm.

The current trial was conducted to determine whether SAPPS could be used to culture *H. diversicolor supertexta* in small tanks. Two commercial, artificial micro particle diets were used and each was tested in the dark and light to determine if natural microalgal related food was affecting results.

MATERIALS AND METHODS

Methodology for SAPPS

Two experimental versions of the commercially available artificial microparticle diet "Plate Powder" were supplied by Adam and Amos Pty Ltd (Mt Barker, Australia). The diets were sieved and only particles of 53- μm or less were used in the trial. The original particle size of diet 1 was quite large and less than 10% of the diet passed through the sieve. However, the original particle size of diet 2 was smaller than diet 1, with over 70% of the particles passing through the sieve. Examination of both diets under a compound microscope at $\times 400$ magnification revealed that

*Corresponding author. Email: 9estott@hotmail.com

many of the ingredients were not finely ground during the initial grinding process when the diets were produced. Large clumping of individual ingredients was observed among particles that failed to pass through the sieve.

A solution of agar was prepared by dissolving 10 mg/mL of agar powder (Kokusan Chemicals; Tokyo, Japan) in boiling, deionized water. Once the temperature of the solution had fallen to 60°C, 100 mg/mL of micro particle diet was added to the solution and the contents were thoroughly mixed. Results from pretrials confirmed that agar by itself (without microparticulate diet) was inappropriate for supporting high growth and survival of postlarval *H. discus discus*.

The resulting solution was then transferred to a MK-7 fine mist sprayer (Culmar; NY, USA) and the temperature was maintained at approximately 50°C. Each plastic plate (dimensions of 22.5 cm × 11.5 cm) was sprayed (individually) after being removed from the experimental tank and allowed to drain for approximately 10 sec. Plastic plates were sprayed by holding them 30 cm away from the sprayer. The entire surface of the plate was subsequently sprayed with solution contained in the MK-7 fine mist sprayer. Pretrials confirmed that the SAPPs methodology did not compromise postlarval survival. Results from pretrials also confirmed that newly metamorphosed postlarvae did not incur mortality via drowning from the fine mist produced by the sprayer and the temperature of the agar/diet solution dropped to safe levels during the process of spraying (as the solution moves through the air and contacts with the wet plastic plates). Each side of the plastic plate was sprayed twice (approximately 0.4 mL of solution per side). Once the spray process was complete, the plate was quickly placed back into the experimental tank.

Spawning and Larval Settlement

Broodstock *H. diversicolor supertexta*, at a ratio of two females to one male, were induced to spawn at Banda Marine Laboratory, Tokyo University of Fisheries, using UV light and temperature shock. After hatching, larvae were reared at three larvae/mL in a 100-L flow-through system with a flow rate of 400 mL/min and a temperature of 23°C. Larvae were deemed competent for settlement when the third tubule on the cephalic tentacle appeared. At this stage larvae were concentrated, counted, and then transferred to the laboratory ready for assignment to the experimental tanks.

Seven days before placing the plastic plates in the experimental tanks, they were preconditioned with juvenile abalone mucus to act as an attachment and settlement inducer. This was achieved by spraying the appropriate diet onto the plastic plates using the method stated earlier. The plates were then placed vertically into a tank with a small number of juvenile *H. discus discus* (20–50 mm) and the tank covered with black plastic to prevent the growth of microalgae. The juvenile abalone were encouraged to graze the diet coated plates and the food was reapplied via the spray system every second day for a period of 5 days.

Two conditioned plates (with the appropriate diet) were hung vertically in each 10-L experimental tank and approximately 2,000 larvae were introduced. Larvae were allowed to settle for 24 h, and then the plastic plates were removed and the number of attached larvae was enumerated for each tank. In this study, abalone larvae were considered to have completed metamorphosis when they had dropped their velum and showed evidence of peristomal shell growth.

Unattached larvae in the experimental tanks were discarded

along with the seawater when the tanks were cleaned. All tank surfaces were scrubbed with a scrubbing brush and washed with freshwater to remove any larvae that had attached. The tanks were then refilled with fresh seawater, ready for the postlarval grow-out phase of the trial.

Post-larval Grow-out

The trial consisted of 4 treatments: Diet 1-dark (D1-D), Diet 1-light (D1-L), Diet 2-dark (D2-D), and Diet 2-light (D2-L), each replicated three times (total of 12 experimental units). Independent tanks were used for replicates. Each experimental unit was assigned a 10-L flow-through tank that was supplied with charcoal filtered water at 250 mL/min. The tank's outer surfaces were painted with black paint and 150 µm was glued over the outlet of the tank to stop dead larvae from being flushed out of the tanks. The tanks to be placed in the dark, D1-D and D2-D, were covered with black plastic. The tanks remaining in the light, D1-L and D2-L, were left uncovered and exposed to a natural lighting regimen (approximately 7,000 LUX during daylight hours).

Food was sprayed onto the plastic plates using the earlier mentioned method, on day 1 (after the 24 h settlement period) and every second day thereafter. Tanks were siphoned every second day and the siphoned water samples were examined and the number of dead postlarvae tallied to calculate percentage survival. Postlarvae from the siphoned water samples were transferred to a glass slide with a pipette and examined to confirm if they were dead. This process was also used to determine the relative proportion of mortality of larvae (no peristomal shell formation) and postlarvae (peristomal shell formation). Tanks were also thoroughly scrubbed twice per week to remove any attached microalgae, etc. At the end of the experiment the total number of postlarvae remaining in each tank was enumerated. This number was compared with previously recorded survival data to validate the accuracy of the methodology.

Post-larvae were sampled twice per week by randomly removing 10 animals from plastic plates in each tank using a soft brush. The size (maximum standard length) of postlarvae was taken using a compound microscope fitted with an ocular eyepiece. Water temperature was measured using a standard thermometer and averaged 25.3°C ± 0.9°C.

Approximate Analysis and Particle Size Determination of the Diets

Proximate analysis of the diets for moisture, crude protein, crude lipid, and crude ash was performed using the methodology outlined in Hernandez et al. (1995).

The particle size of the artificial diets was determined by randomly extracting 40 particles and measuring the size under a compound microscope fitted with an ocular eyepiece, at ×100 magnification.

Statistical Analysis

Statistical analysis was conducted using the computer package SYSAT (SPSS Inc., IL USA). Before conducting statistical ANOVAs, normality was confirmed using normal probability plots and homogeneity of variance was validated utilizing Levene's test. Data was transformed where necessary and a one-way ANOVA performed. Differences in means were determined using Tukey's test.

RESULTS

The mean composition of the diet 1 and diet 2 were similar, with exception to crude lipids (Table 1). Crude lipid was 21.0% for Diet 1 compared with 13.8% for Diet 2. The particle size of diet 1 was slightly larger than diet 2: $34 \pm 11 \mu\text{m}$ compared with $31 \pm 6 \mu\text{m}$.

Attachment of larvae in the different treatments was variable and patchy on the plates in the experimental tanks. Final mean settlement of larvae was 241 ± 142 , 173 ± 87 , 333 ± 56 and 195 ± 60 in D1-D, D1-L, D2-D, and D2-L respectively (Table 2).

Mortality of postlarvae in the different treatments was high in the first week, ranging from 55.4% to 74.8% (Fig. 1). The majority of these mortalities were observed to have no peristomal shell growth. Thus, a high percentage of the mortalities in the first week were larvae that had attached but failed to complete metamorphosis. Very little mortality occurred after the first week and final mean mortality of postlarvae in the different treatments ranged between 14.8% and 31.4%.

The daily growth rate of postlarvae in all treatments increased in the ensuing weeks (Fig. 2). The final mean size of post larvae was significantly higher ($P < 0.05$) in D2-D ($1,695 \pm 148 \mu\text{m}$) and D2-L ($1,752 \pm 134 \mu\text{m}$) compared with that of D1-D ($1,488 \pm 153 \mu\text{m}$) and D1-L ($1,512 \pm 181 \mu\text{m}$).

DISCUSSION

H. diversicolor supertexta postlarvae grew well using SAPPS, regardless of light, suggesting that they can grow without the requirement of light or photosynthetic live food. Plastic plates in D1-D and D2-D were kept in the dark to have been relatively free of microalgae. Additional measures to limit microalgal growth in the experimental tanks were also included (e.g., supplying tanks with charcoal filtered water and scrubbing them twice weekly). Thus, postlarvae were able to gain adequate nutrition via SAPPS and those fed diet 2 grew at over $50 \mu\text{m/day}$.

Post-larvae that were grown in the dark may have gained exogenous nutrition from the artificial diet, bacteria that proliferated on the plates and mucus deposited by the juvenile abalone. Takami et al. (2000) found that *H. discus hannai* shift from yolk energy source to particulate food by the time they reach $500 \mu\text{m}$. Takami et al. (1997) reported that all *H. discus hannai* larvae that were raised on an unsuitable diatom biofilm died before they reach $500 \mu\text{m}$ and all those raised exclusively on abalone mucus died before they attained a size of just over $700 \mu\text{m}$. Postlarvae over 1 mm in length require high levels of digestion of diatoms in addition to any extracellular substances for favorable growth (Kawamura 1996). Thus, in this study, postlarval abalone fed diet 2 grew to about

$1,700 \mu\text{m}$ and the growth rate doubled from week 1 to week 4 (approx. $30 \mu\text{m}$ to $60 \mu\text{m}$). Therefore, postlarvae were most likely gaining a high level of nutrition from the artificial micro diet. Visual observations also confirmed that food was in the gut and mortality after week 1 was minimal. However, the influence of bacteria on growth and survival of postlarval abalone was not determined.

Post-larval abalone cultured in the dark grew as well as those grown in the light. Photosynthetic food that may have grown on the plates, provided postlarvae no advantages in terms of growth and survival. It seems that abalone in this trial had no requirement for light. However, it is also necessary to investigate the opposite scenario because complete, continuous darkness could potentially offer postlarval abalone advantages in terms of growth and survival. Adult and juvenile *Haliotis* spp. mainly feed at night (Ino 1943, Sakai 1962, Uki 1981) and grow better when cultured in the dark (Ebert & Houk 1984). However Velez-Espino (1999) found that there was no evidence of nocturnal feeding habits in postlarvae. Abalone postlarvae remained attached to the plastic plates but become increasingly sensitive to light after they develop their first respiratory pore (Hahn 1989). Thus, postlarval feeding most likely takes place regardless of light or time of day. Darkness at this trial provided no advantages in terms of growth and survival.

The final mean size of postlarvae fed diet 2 was greater than that of postlarvae fed Diet 1. Proximate composition of the diets was similar, apart from a higher percentage of crude lipids in Diet 1, compared with Diet 2. Uki et al. (1985) found that growth performance of *H. discus hannai* was maximized at 5% crude lipid inclusion in an artificial diet. Other species such as *H. tuberculata* have an optimum growth rate at levels as low as 3.11% crude lipid (Mai et al. 1995). In the current trial, the artificial diet was supplied by a commercial manufacturer and no information was made available regarding the ingredients or binders incorporated into the diet. The only obvious difference after proximate analysis was lipid content. Total crude lipid in diet 1 was 21% compared with only 13.8% in diet 2 and this high inclusion of lipid, although not conclusive, may have negatively affected postlarval growth. Processing the artificial micro particle diet for application via SAPPS may have affected the original gross composition or nutritional balance. The sieving process resulted in selection of small particles ($< 53 \mu\text{m}$), whereas larger particles were excluded. The original particle size of diet 1 (before sieving) was much larger than diet 2 and this resulted in high particle retention when sieving with a $53\text{-}\mu\text{m}$ sieve. This may have caused higher selection of particles that contained high levels of lipids. However, because the crude lipid content of the diets before sieving was not determined and a list of ingredients and methods used to make the diet were not available, the exact reason for such a high level of lipids in the diet remains unclear.

In future, it is recommended that ingredients incorporated into diets that are applied using SAPPS, be thoroughly ground. Additionally, after processing the final diet, if the particle size of the diet is over $53 \mu\text{m}$, the diet should be ground down further to create a finer particle size before sieving. Artificial abalone diets that have been made for juvenile or adult abalone can also be used in SAPPS provided they are ground down to a fine powder.

Extremely high postlarval mortality in the first week of the trial in all treatments was due to a high rate of unsuccessful metamorphosis of larvae. Many of the larvae attached to the plates, dropped their vellum but failed to deposit peristomal shell. These larvae died before the end of the first week. However, larvae that com-

TABLE 1.

The size and composition of the two test diets.

	Diet	
	1	2
Mean particle size (μm) \pm SD	34 ± 11	31 ± 6
Moisture (%)	11.1	9.8
	On dry matter basis (%)	
Crude protein	31.5	30.8
Crude lipid	21.0	13.8
Crude ash	7.5	7.0

TABLE 2.

Results of a 4-week feeding trial for post-larval *H. diversicolor supertexta* fed two different artificial micro particle diets in the light and dark.

Treatments	Mean Number Settled \pm SD	Final Mean Measurements		
		Size \pm SD (μ m)	Daily Growth Rate \pm SD (μ m)	Survival Rate \pm SD (%)
Diet 1 (D)	241 \pm 142	1488 \pm 153 ^a	43 \pm 4	18.8 \pm 3.7
Diet 1 (L)	173 \pm 87	1512 \pm 181 ^a	44 \pm 5	20.4 \pm 14.8
Diet 2 (D)	333 \pm 56	1695 \pm 148 ^b	51 \pm 4	31.4 \pm 1.2
Diet 2 (L)	195 \pm 60	1752 \pm 134 ^b	53 \pm 4	14.8 \pm 5.1

Different superscripts in the same column denote difference in means ($P < 0.05$)

pleted metamorphosis were in high densities in small patches on the plates. Thus, it is possible that the plates were not adequately conditioned with mucus due to the fact that the number of juvenile abalone that were placed into the tanks to condition the plates with mucus for larval settlement, were too low. It was also observed that food sprayed on the plates for juvenile abalone during the conditioning period was not heavily grazed. Mucus was only deposited on the regions of the plastic plates where juvenile abalone grazed. Other regions of the plastic plates (with no mucus) larvae attached at lower densities and most of these larvae failed to complete metamorphosis. In a previous trial, *H. discus discus* larvae attached to blank plastic plates at a rate of up to 20%, but successful metamorphosis occurs in less than 5% of the larvae (Stott et al. In press). In the same study, attachment and metamorphosis was increased to about 80% by spraying freeze-dried algal powders (*Spirulina platensis* or *Chlorella vulgaris*) onto plastic plates and then conditioning them with abalone mucus by allowing juvenile *H. discus discus* to graze the plates. Takami et al. (1997) reported that *H. discus hannai* settled on abalone mucus conditioned plastic slides at a rate of 48.7% and that all postlarvae died by the end of the third week. This documents a much higher rate of attachment than the current trial but no larvae completed metamorphosis (and only 10% attached) in a control group that was offered no settlement stimulus. Additionally, as with our previous trial, the same species of juveniles as the larvae were used to condition plates in the mucus treatment.

It is possible that mucus from juvenile *H. discus discus* was not

a strong attachment and metamorphosis inducer for larval *H. diversicolor supertexta*. Seki and Kanno (1981) found that larval *H. discus hannai* settled on mucus from 4 different *Haliotides* but not on mucus from a number of mollusc from different genus to *Haliotis*. However, in that study, percentage settlement was not determined and *H. diversicolor supertexta* was not tested. *H. diversicolor supertexta* is a small species of abalone that spawns sporadically over the summer period whereas *H. discus discus* is a relatively large species of abalone that spawns usually once in autumn (Hahn 1989). Attachment and metamorphosis rates of larval abalone may be improved by using juvenile abalone from the same species for conditioning plates.

After the first week, postlarval survival was very high. Even though the average water temperature was over 25°C, the use of artificial diets did not seem to adversely affect culture conditions. Hahn (1989) suggests that most abalone farmers stop using artificial diets when water temperatures rise above 24°C. The methodology for SAPPs results in the artificial micro particle diet being coated with agar. Stott et al. (2003b) found that nitrogen leaching from an artificial micro diet after 12 h immersion was nearly reduced by half when binding the diet in an alginate gel compared with adding the diet directly to the water column. Although this was not quantified in this trial, it is likely that binding the diet in an algal gel would protect it from bacterial degradation and leaching that may potentially adversely affect water quality.

There is a need for greater research in this area before modi-

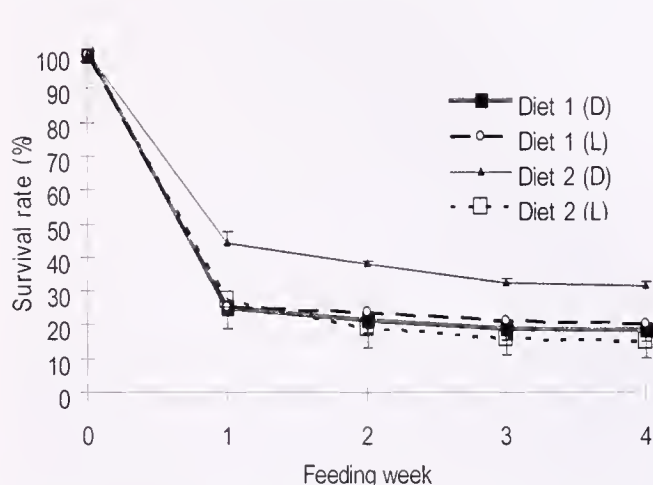


Figure 1. Survival rate (\pm SD) of postlarval *Haliotis diversicolor supertexta* fed two different artificial micro particle diets in the dark and light over a 4-wk period.

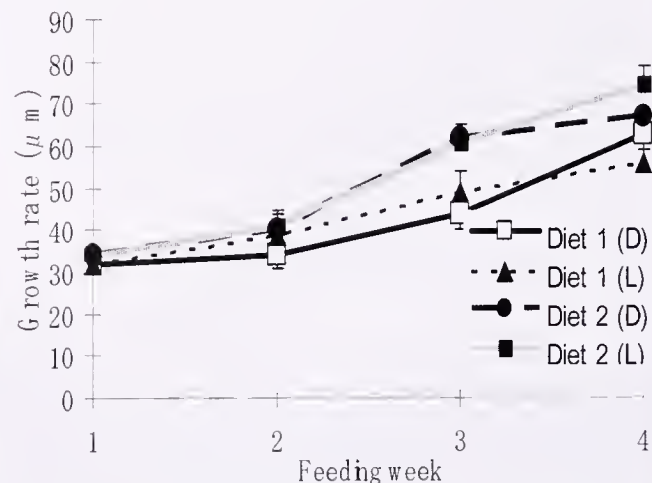


Figure 2. Mean growth rate (μ m)/day (\pm SD) for postlarval *Haliotis diversicolor supertexta* fed two different artificial micro particle diets in the dark and light over a four week period.

fying SAPPs for use in industry. It will be necessary to determine optimum concentration of agar as well as feeding frequency. A major disadvantage of SAPPs is that it is an extremely intensive method and spraying the plates requires much effort that may translate into high labor costs. It would be beneficial to spray plates as infrequently as possible without adversely affecting post-larval growth and survival. SAPPs allows application of artificial diet to wet plates but some of the diet is lost when replacing plates into the tanks.

In the near future, commercial trials of SAPPs will be con-

ducted using state of the art spraying technology and incorporating new larval settlement technology.

ACKNOWLEDGMENTS

The authors thank the staff from Banda Marine Laboratory for their assistance throughout the trial. Many thanks to Joel at Adam and Amos Pty. Ltd. (Mt Barker, Australia) for supplying the artificial diets ("Plate Powder") used in this trial. Finally, thanks to Ronald Mitchell and anonymous colleagues for reviewing the manuscript.

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EFFECT OF TEMPERATURE ON THE EARLY DEVELOPMENT OF *HALIOTIS DIVERSICOLOR* REEVE

JUNYI LU,* QIANG LIN, YANYAN SUN, JUNQING SHENG AND QINGXIANG CHEN

Institute of Aquatic Economic Animals and Guangdong Provincial Key Laboratory for Aquatic Economic Animals, Zhongshan University, Guangzhou 510275, China

ABSTRACT The effect of temperature on the early development of *Haliotis diversicolor* Reeve was studied as a strategy to define the optimum temperature range for experiments on early developmental conditions of this species. The survival rate of *Haliotis diversicolor* Reeve of different stages of early development varied significantly at different temperatures (17°C, 21°C, 24°C, 27°C, 31°C, and 33.5°C) ($P < 0.01$). In the cleavage period, the survival rate was 1.7% at 17°C and 99.4% at 27°C. When the water temperature was 33.5°C the survival rate was 17.9%, much lower than 97.9% at 31°C. The other 3 embryonic periods, including embryonic development, postembryonic development, and emergence stage of the 1st breathing pore, also showed the similar trends with variations in temperature. When temperature increased between 18°C and 30°C, the early developmental rate accelerated, the developmental time shortened and survival increased. The relationship between the developmental rate or the developmental time and water temperature can be expressed by the following formula: $Y = 0.0541 / (1 + 5.8381e^{-0.2532t})$ ($r = -0.7014$, $P < 0.05$). The first trochophore needed 20 h to hatch out with an instantaneous hatching rate of 0.2% at 17°C and the total hatching rate reached 2.5% at 28 h. When temperature was maintained at 27°C the time of first trochophore hatching was at 8 h postfertilization (h.p.f.) with an instantaneous hatching rate of 10.3% and the highest total hatching rate was 96.4% at 22 h.p.f. when the incubation ended. The total hatching rate declined to 85.3% at 33.5°C. The settlement of trochophore was also affected by temperature. The trochophores started to settle at 65 h.p.f. with a low settlement rate of 0.5% only at 17°C and started to settle at 55 h.p.f. with the highest settlement rate of 53.2% at 27°C and only 1.2% at 33.5°C. The results showed that the optimum water temperature for artificial reproduction and seed breeding of *H. diversicolor* was 24°C to 28°C.

KEY WORDS: *Haliotis diversicolor* Reeve, early development, temperature

INTRODUCTION

Haliotis diversicolor Reeve is a species of abalone especially popular in South China because of its appropriate size and unique flavor. The reproduction and breeding of abalone is the foundation of commercial culture. The reproductive cycles of several important abalone species have been studied. The reproductive biology of the Donkey's ear abalone, *H. asinina* Linné were studied, including the sex ratio, initial size at sexual maturity, spawning period, fecundity, and so on (Capinpin et al. 1998). Jarayabhand et al. (1995) reported on the embryonic and larval development of *H. ovina*. Early development showed differences in developmental stages between *H. asinina* and *H. ovina* in Thailand, physico-chemical factors having important effects on the growth and survival. (Jarayabhand & Paphavasit 1996). It has been reported that salinity plays an important role in the early development of oysters (Tan & Wong 1996) and the juvenile abalone (Chen & Chen 2000). The hatching, larval growth, survival, and settling of oysters were reported to be affected by salinity (Liu et al. 1992, Madrones-Ladja et al. 2002a, Nell & Holliday 1988, Tan & Wong 1996). Salinity, micro algal diet, and rearing conditions also affected gonad maturity and fecundity, early development, and larval growth of the window-pane shell (Madrones-Ladja et al. 2002a, Madrones-Ladja et al. 2002b). Dos Santos & Nascimento (1985) reported that the normal early development of the mangrove oyster was influenced by gamete density, salinity, and temperature. The early development of *H. diversicolor* was suggested to be divided into 4 periods, which were cleavage, embryonic development, postembryonic development, and emergence stage of the 1st breathing pore (Lu et al. 2001). This study investigates the effect of temperature on the early development, developmental rate, hatching, and settlement of the trochophore.

MATERIALS AND METHODS

Materials and Water Treatment

The fertilized eggs used in the incubation experiment were collected by the following procedure: 2+-year-old parental abalone (shell length of 50–65 mm and body weight of 41.5–62.5 g) were induced to spawn by temperature stimulation and then the eggs were artificially fertilized (Lu et al. 2001).

To test the variance in the embryonic development of *H. diversicolor* at different water temperatures, we adopted 6 different temperature ranges: 17°C, 21°C, 24°C, 27°C, 31°C, and 33.5°C ($\pm 0.5^\circ\text{C}$). The seawater used in the experiments was treated by double sand-filtration (particle diameter, 0.05–0.10 mm) and bio-filtered at salinities of 28‰ to 35‰ and at pH of 8.2–8.5.

Experiments

All experiments were conducted in triplicate at six different water temperatures.

Embryonic Development

The fertilized eggs were incubated in 500 mL glass beakers under different water temperatures. After fertilization, 2-mL samples, which were at a density about 10 eggs/mL, were taken every 3–5 min for microscopic examination to observe the cleavage progress. One hour later, samples were taken every 10 min for observation of embryonic development, and larvae were counted every 0.5 h.

Trochophore Settlement and Survival

One thousand trochophores were stocked in 150-mL glass beakers at a density of 10 individuals/mL at different temperatures. Samples were examined to observe the shaping of larval organs

*Corresponding author. E-mail: ls61@zsu.edu.cn

TABLE 1.

Arithmetic mean (\pm SD) of the variance of survival rate (%) of different stages in the early development at 6 temperature levels (17, 21, 24, 27, 31 & 33.5 °C).

Temperature (°C)	Fertilized Eggs Stage	Cleavage Stage	Embryonic Stage	Post-embryonic Stage	Emergence Stage of the 1st Breathing Pore
17	0.665 \pm 0.031 ^d	0.017 \pm 0.001 ^c	0.727 \pm 0.100 ^b	0.250 \pm 0.015 ^f	0.000 ^d
21	0.788 \pm 0.041 ^c	0.649 \pm 0.125 ^c	0.765 \pm 0.062 ^c	0.296 \pm 0.018 ^c	0.845 \pm 0.037 ^b
24	0.915 \pm 0.030 ^b	0.885 \pm 0.015 ^b	0.929 \pm 0.012 ^{ab}	0.931 \pm 0.032 ^b	0.928 \pm 0.042 ^a
27	0.996 \pm 0.029 ^a	0.994 \pm 0.042 ^a	0.995 \pm 0.042 ^a	0.996 \pm 0.015 ^a	0.961 \pm 0.085 ^a
31	0.978 \pm 0.076 ^a	0.979 \pm 0.070 ^a	0.858 \pm 0.010 ^b	0.743 \pm 0.075 ^c	0.831 \pm 0.020 ^b
33.5	0.543 \pm 0.061 ^c	0.184 \pm 0.071 ^d	0.217 \pm 0.067 ^d	0.382 \pm 0.086 ^d	0.375 \pm 0.085 ^c

using a binocular dissecting microscope, every 2 h, until all of them metamorphosed to settle or die.

Statistical Analysis

One-way ANOVA was used to examine the effects of temperature on survival of different stages in the early development, and regression analysis was used to test the relationship between developmental rate and temperature. When necessary, data were treated and assessed statistically by using 11.5 SPSS (Statistical Program for Social Sciences) computer software.

RESULTS

Effect of Temperature on the Early Development of *H. diversicolor*

The survival rate of different stages in the early development varied greatly at temperatures of 17°C, 21°C, 24°C, 27°C, 31°C, and 33.5°C ($P < 0.01$). In the cleavage period the survival rate was 1.7%, 65.1%, 88.5%, 99.4%, and 97.9% at the temperature of 17°C, 21°C, 24°C, 27°C, and 31°C, respectively. The results indicated that 27°C was the optimum temperature for the early development because of its highest survival rate of 99.4%, and the next highest was 97.9% at 31°C. However, when the water temperature increased 33°C to 34°C the survival rate decreased to 17.9%. In addition, the survival rate of other periods such as embryonic development, postembryonic development, and emer-

gence stage of the 1st breathing pore (see Lu et al. 2001) also showed similar trends at water temperature of 17°C to 34°C, and temperatures between 24°C and 27°C also had higher survival than the others (Table 1). The survival rate at the same temperature among different stages didn't show significant variance ($P > 0.05$).

Effect of Instantaneous Water Temperature on the Early Developmental Rate

The early developmental time and its rate were influenced by instantaneous water temperature. The early developmental time shortened when the water temperature increased from 16°C to 32°C. When the temperature increased to 34°C, the developmental rate slowed down and a longer time was needed to develop. The developmental time was directly related to water temperature when temperatures ranged from 16°C to 30°C. In contrast, the developmental rate was inversely related to water temperature between 16°C and 30°C (Fig. 1). The relationship between the developmental rate or the developmental time and temperature can be expressed by the logistic formula:

$$Y = 0.0541 / (1 + 5.8381e^{-0.2532t}) \quad (r = -0.7014, P < 0.05).$$

Y: developmental rate or the developmental time t: temperature

Effect of Temperature on the Hatches of Trochophores

Under the artificial breeding conditions, the incubation of the fertilized eggs of *H. diversicolor* was mainly affected by temperature. The first trochophore required 20 h to hatch out with an instantaneous hatching rate of 0.2%, and the total hatching rate reached 2.5% at 28 h postfertilization (h.p.f.), and the final survival rate was 16.0% at a mean temperature of 17°C. The trochophores started to hatch out at 10 h. p. f. at 21°C and 8 h.p.f. at 24°C. When the temperature was maintained at 27°C, the time was 8 h with an instantaneous hatching rate of 10.3% for the hatching out of the first trochophore, and this produced the highest total hatching rate of 96.4% and a survival rate of 81.85% at 22 h. p.f. The total hatch rate declined to 94.9% at 31°C and 85.3% at 33.5°C

TABLE 2.

The time of the first trochophore of hatching out and instantaneous hatching rate.

Temperature (°C)	17	21	24	27	31	33.5
Time (h)	20	10	8	8	6	8
Instantaneous hatching rate (%)	0.2	1.5	6.8	10.3	8.1	11.5

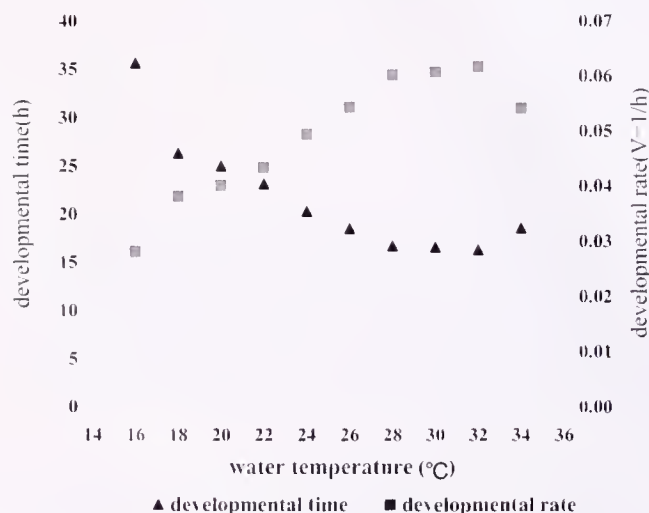


Figure 1. The relationship between the early developmental rate or the early developmental time and water temperature.

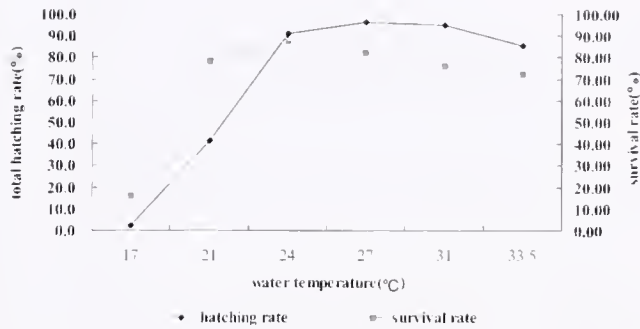


Figure 2. The hatching rate and survival rate of trochophores at six temperature levels.

with a survival rate of 76.08% and 72.33%, respectively (Table 2, Fig. 2 and Table 3).

Effect of Temperature on the Settlement of Trochophores

Temperature also affected the settlement of trochophores. The trochophores started to settle at 65 h, with a low settlement rate of 0.5%, when the mean temperature was 17°C. When the water was 21°C, the trochophores started to settle earlier at 55 h, and the number of settled individuals increased to 17.4%. The settlement rate was 53.2% at 27°C; 34.9% at 24°C; 29.1% at 31°C and 1.2% at 33°C to 34°C (Fig. 3). In addition, results showed that the instantaneous settling rates were 5.30%, 85%, 168%, 87%, and 5% at 17°C, 21°C, 24°C, 27°C, 31°C and 33.5°C, respectively (Table 4).

DISCUSSION

We found that salinity was relatively steady at 28‰ to 35‰ and pH was 8.2–8.5 whereas temperature varied from 12°C to 32°C in the artificial breeding of *H. diversicolor* (unpublished), and it had also been proven that temperature greatly influenced early development, seed breeding, and growth of *H. diversicolor* (Chen & Chen 1999). The survival rate of different stages in the early development showed significant difference within different temperature ranges ($P < 0.01$). Water temperature played an important role in the fertilization of eggs and resulted in the failure of fertilization at lower (16°C to 22°C) or higher (33°C to 34°C) temperatures, which was at a rate of 33.5% and 45.8%, respectively. According to the observation, low temperature (17°C) can also terminate the development of the cleavage stage of the fertilized eggs at a rate of 97%. From Table 1 and Figure 1 we can see that the final survival of juveniles was higher at 24°C to 31°C than that at 17°C and 33.5°C with shorter developmental time and faster developmental rate. Generally speaking, the shorter time of the incubation and hatching, the shorter time the individuals were exposed to adverse environmental factors such as parasitic fungi or predation by copepods.

Within the optimum range of temperature, the higher the tem-

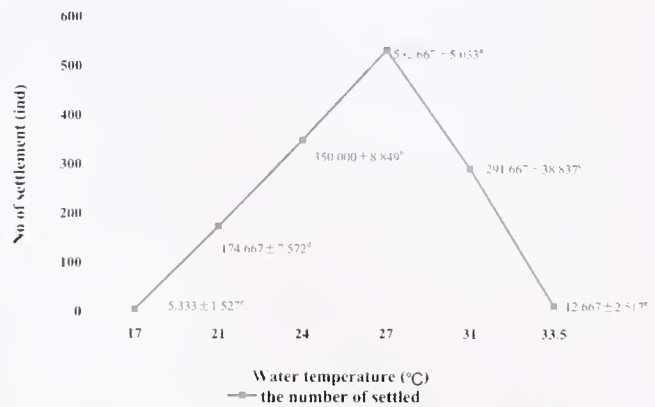


Figure 3. Variation of the settlement of trochophores at six temperature levels.

perature, the shorter the developmental time, which was similar to that of fish such as eel *Anguilla japonica* (Xie et al. 1995), *Silurus glodotovi meridionalis* Chen (Chen et al. 1998), *S. asotus* Linnaeus (Xiao et al. 1998) and *Megalobrama hoffmanni* (Ye et al. 1998), and snail *Babylonia formosae habei* (Zheng et al. 2000).

The first trochophore of *H. diversicolor* hatched out at 20 h.p.f. at 17°C, 10 h.p.f. at 21°C, 8 h.p.f. at 24°C and 27°C, 6 h.p.f. at 31°C, and 8 h.p.f. at 33.5°C (Table 2). Jarayabhand et al. (1995) reported that the trochophores of *H. ovina* hatched out at 7–8 h and trochophores of *H. asinina* hatched out at 5 h (Jarayabhand & Paphavasit 1996) at the average temperature of 29°C. The latter species was a little earlier than the former in hatching out.

Planktonic marine invertebrate larvae experience high mortality rates. Processes during these early vulnerable stages of development are an important determinant of the dynamics of marine invertebrate populations (Drent 2002). Those that hatched out at temperatures lower than 18°C or higher than 32°C had lower swim-up rates and settling rates. When the temperature exceeded 32°C the hatching rate was relatively high, but more trochophores were malformed and incapable of swimming or settling, and this prolonged the duration of settling and finally the animals died of fatigue. This was similar to results obtained on *H. discus hamai* Ito (Chen et al. 1977).

According to the results of the experiments, the optimum temperature for incubation and seed production was 24°C to 28°C and this proved feasible in large-scale artificial breeding of *H. diversicolor*. We got mass production of juveniles (shell length at 1.8–2.5 cm) at densities of 4,806 ind/m² in 2001 (Lu et al. 1999).

ACKNOWLEDGMENT

This study was funded by the National High Technology Research & Development Program of China (863 Program) (2002AA624010) and Key Science & Technology Program of Guangdong Province of China (99M03201G).

TABLE 3.

Arithmetic mean (\pm SD) of the hatching rate and survival rate of trochophores at six temperature levels.

Temperature (°C)	17	21	24	27	31	33.5
Hatching rate	2.477 \pm 0.127 ^a	41.033 \pm 1.365 ^d	91.100 \pm 1.778 ^b	96.467 \pm 0.907 ^a	94.867 \pm 1.550 ^a	85.267 \pm 2.369 ^c
Survival rate	15.967 \pm 1.258 ^c	78.105 \pm 1.513 ^c	87.167 \pm 1.405 ^d	81.767 \pm 1.419 ^b	76.033 \pm 1.266 ^c	72.200 \pm 1.931 ^d

TABLE 4.

The time of the first trochophore to settle and Arithmetic mean (\pm SD) of instantaneous settling rate.

Temperature ($^{\circ}$ C)	17	21	24	27	31	33.5
Time (h)	65	55	55	55	60	55
Instantaneous settling rate (%)	5.033 \pm 0.862 ^d	30.067 \pm 1.721 ^c	84.833 \pm 3.014 ^b	168.333 \pm 3.786 ^a	87.667 \pm 3.215 ^b	4.967 \pm 1.050 ^d

Note: Data of instantaneous settling rate (%) are Arithmetic mean and Sample standard deviation.

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FATTY ACID COMPOSITION OF EGGS DERIVED FROM CONDITIONED AND WILD CAUGHT GREENLIP ABALONE BROODSTOCK (*HALIOTIS LAEVIGATA*)

SABINE DAUME^{1*} AND STEPHEN RYAN^{1,2}

¹Department of Fisheries, Research Division, PO Box 20, North Beach, WA 6920, Australia;

²Great Southern Marine Hatcheries, PO Box L34, Little Grove, Albany WA 6330, Australia

ABSTRACT In the present study, the fatty acid profiles of eggs spawned from conditioned and wild caught greenlip abalone broodstock (*Haliotis laevis*) were compared. Lower proportions of polyunsaturated fatty acids (12% and 9%) compared with monounsaturated (44% and 45%) and saturated fatty acids (32% and 34%) were present in eggs of conditioned and wild caught broodstock respectively. Relative amounts of individual saturated and monounsaturated fatty acids were similar in eggs of both wild caught and conditioned broodstock. The relative proportions of some polyunsaturated fatty acids of the broodstock diets were reflected in the eggs and varied between batches of conditioned and wild broodstock. Formulated feed has high linoleic acid (18:2n-6) and the proportion of this fatty acid was 10 times higher in eggs spawned from animals fed formulated feed than in eggs of wild caught broodstock. Eggs derived from wild broodstock, which feed preferentially on red seaweeds, showed about twice as much arachidonic acid (20:4n-6) compared with eggs from conditioned broodstock, which were supplied with a formulated diet low in arachidonic acid. Arachidonic acid is a major precursor of prostaglandins, which play a vital role in molluscan reproduction. In this study, the levels of ARA were higher in eggs than in the respective diets of the broodstock, except of the *Gracilaria* diet, indicating that ARA was preferentially incorporated into the eggs of broodstock feeding on formulated feed and *Ulva australis*. Results from the present study demonstrate that 3 mo of feeding on an altered diet is sufficient to alter the fatty acid profile of the eggs. This study shows high variability in diameter, weight, and biochemical composition of eggs between hatches and spawning seasons. We present initial evidence that smaller eggs had higher lipid content with a higher larval hatch rate than larger eggs. In addition, larval settlement was negatively correlated with egg moisture.

KEY WORDS: abalone eggs, arachidonic acid, fatty acids, *Haliotis laevis*, lipid

INTRODUCTION

Abalone farming is a well-established industry in many countries worldwide but is still in early development in Australia. Successful conditioning of broodstock is a crucial step for selective breeding programs to develop this growing industry. Gonad maturation and larval settlement success of abalone are known to vary seasonally (Webber 1970, Slattery 1992). However, large variability in spawning events, hatchability, and larval and juvenile survival rates has been observed during the same season between hatches and hatcheries in Australia.

Formulated diets designed to maximize growth rates are not necessarily adequate to maintain viable, high quality eggs and larvae from captive broodstock. There is growing evidence that specific dietary lipids play an important role in gonadogenesis of abalone (Uki & Watanabe 1992, Nelson et al. 2002). Recently, Nelson et al. (2002) showed that variations of the C₂₀ polyunsaturated fatty acid (PUFA) in the digestive gland and foot tissue of *Haliotis fulgens* over the year coincided with variation in their macroalgal diets. This study indicated that abalone tissues directly reflect their diets. Furthermore, the authors suggested that arachidonic acid (ARA) is an essential fatty acid for the abalone *H. fulgens*. They concluded that essential fatty acids are derived from the algal diet and are most likely important in cyclical gonad development.

Studies of changes in biochemical composition such as fatty acids in mollusc eggs are scarce. Soudant et al. (1996) established that PUFA composition of great scallop (*Pecten maximus*) gonad was related to the fatty acid composition of the diet. Gallager & Mann (1986) reported a significant correlation between the initial egg lipid content and survival of the bivalve *Crassostrea virginica* larvae. In addition, the growth of *Ostrea edulis* was affected by the

amount of lipid present in larval and spat tissue (Laing & Millican 1986). Changes in biochemical composition during larval development were reported for scallops (Whyte et al. 1990, Farias et al. 1998), oysters (Ferreiro et al. 1990) and abalone (Litaay et al. 2001). Lipids in abalone eggs are known to partially fuel the metamorphosis of the larvae (Jaekle & Manahan 1989). Variations in lipid content and fatty acid profile have been shown, by Nelson et al. (2002), to be related to seasonal temperature fluctuations and variations of the diet may ultimately affect the success of the progenies.

In this study we determine the fatty acid profiles of eggs spawned from conditioned broodstock (feeding on formulated feed) and wild caught broodstock of *Haliotis laevis*. In addition, 3 conditioning diets are tested with one group of female broodstock continually being fed the commercially used broodstock conditioning diet until spawning, the second group fed the green alga *Ulva australis* and the third group fed a red seaweed diet (*Gracilaria flagelliformis*, *Champia parvula*) for the last 3 mo before spawning, to examine if a dietary change affects the fatty acid composition of the eggs. The aim is to determine how the lipid quality of the broodstock diet affects the fatty acid composition of *H. laevis* eggs and to evaluate the biologic consequences such as larval hatch rate, larval survival, and settlement performance. This information contributes to the knowledge on abalone broodstock quality as well as larval nutrition.

METHODS

Broodstock Collection and Maintenance

All broodstock was collected from the Southern Ocean in Western Australia, from waters between Augusta (115°16'E; 34°32'S) and Hopetown (120°13'E; 33°95'S). The term "conditioning" describes the process to induce gonad development, to the state of spawnability, in adult abalone outside of the normal

*Corresponding author. E-mail: sdaume@fish.wa.gov.au

spawning season. The conditioned broodstock were held indoors at a commercial abalone farm (Great Southern Marine Hatcheries, Albany, Western Australia-GSMH) for a period of 18–20 mo prior to this study. These animals were maintained in round plastic tubs (ca. 30 L volume) fitted with a central standpipe and airstones. The seawater was provided at a flow rate of $1-1 \times L \text{ min}^{-1}$ at constant water temperature of $17.0^\circ\text{C} \pm 0.2^\circ\text{C}$ ($\pm\text{SE}$). Animals were fed daily in excess with a formulated conditioning diet (Adam & Amos Foods Pty Ltd, South Australia). Conditioned broodstock were on average $137 \pm 7.2 \text{ mm}$ ($\pm\text{SE}$) in shell length and had a total weight of $461 \pm 67.4 \text{ g}$ ($\pm\text{SE}$). The wild caught broodstock were larger and averaged $169 \pm 2.9 \text{ mm}$ ($\pm\text{SE}$) and $745 \pm 45.9 \text{ g}$ ($\pm\text{SE}$).

Fatty Acid Composition of Broodstock Diets and Feed Intake

Samples of all abalone diets (seaweeds and formulated feed) were taken during the study and the fatty acid composition determined. Feed intake of the conditioned broodstock was determined daily over a 2-mo period.

Egg Sample Collection and Preparation

Broodstock was induced to spawn with ultraviolet-irradiated water. Egg samples were collected from a routine operation at GSMH over 2 spawning seasons (1st season: November 2001 to January 2002, 2nd season: October 2002 to December 2002). Seven batches of conditioned and 12 batches of wild caught broodstock were compared. Two additional batches derived from selected conditioned female broodstock, previously fed on the formulated feed, were fed on a mix of red seaweed (*Champia parvula*, *Gracilaria flagelliformis*) or *Ulva australis* for 3 mo prior to spawning. Approximately 6 g wet weight of eggs (ca. 400,000 eggs) was collected per batch. Samples were filtered onto pre-weighed glass fiber filters (Whatman GF/C, 4.7 cm diameter) and washed 3 \times with 0.5 M ammonium formate to remove residual salts. Samples were labeled, frozen in liquid nitrogen, stored at -70°C and freeze-dried (Edwards Modulyo Model E2M-18).

Egg Diameter and Offspring Performance

The diameter (egg cytoplasm and vitelline layer) of 30 unfertilized eggs was measured under an inverted microscope at $\times 400$ magnification and averaged for each batch. Eggs were collected within 30 min after spawning started and measured immediately ca. 10–15 min after collection without using any fixative.

Fertilization and hatch rates, larval survival and estimates of settlement, as well as survival at 2-wk postsettlement were recorded for seven of these batches.

Fertilization

Eggs of each batch (1 female abalone) were fertilized with 5–10 sperm per egg for 15 min. Eggs were washed in filtered seawater for 15 min after fertilization to remove excess sperm and to avoid polyspermy. The fertilization rate of each batch was determined by counting the eggs that were dividing after 1 h (indicating that eggs have been successfully fertilized) in three 1-mL samples and expressing these as a percentage of total number of eggs in the batch.

Hatch Rate

Eggs of each batch were placed into separate hatch tubs and formed a monolayer on the bottom of the tub. All tubs were set up

with low water flow and low aeration. The water was turned off ca. 2 h before hatch out started. The hatch rate (expressed as percentage) was determined by counting the number of unhatched eggs in three 1-mL samples, calculating the total of unhatched eggs, and subtracting these from the total number of successfully fertilized eggs.

Larval Survival

Larvae were reared in 300-L conical fiberglass tanks at a stocking density of up to 20 larvae per milliliter. All tanks were set up with low aeration and water flow after the larval shell was completely formed. The survival at the end of the larval rearing period (ca. 6 days at 18°C) was determined by counting the number of larvae in three 1-mL samples at the time of settlement multiplied by the total volume of the larval tank and dividing them by the total number of larvae successfully hatched.

Settlement

Larvae were settled on commercially used PVC settlement plates (40 \times 60 cm) covered by *Ulva lens*, which was cultured on the plates for 2–3 wk prior to larval settlement. Larvae were released into each of three semicommercial fiberglass tanks at a standard density of 100,000 (0.2 larvae mL^{-1}). Settlement tanks were ca. 490 L and set up with three baskets holding 20 settlement plates each. Settlement rate of each batch was determined 48 h after larval release. The term "settlement" describes the permanent attachment of the larvae to the substrate after shedding of the velum to complete metamorphosis. Postlarvae were counted again after 2 wk to determine their survival.

Fatty Acid Analyses

Samples (abalone eggs and diets) were analyzed at the State Chemistry Laboratory, Werribee, Victoria, Australia. Lipid was extracted from the samples using a modified Bligh Dyer method (Bligh & Dyer 1959). The fatty acid profiles were determined by gas chromatography (Varian Model 3400) with a BPX-70 column (SGE Australia) on the methyl esters of the extract from each sample.

Data Analyses

Statistical analyses were carried out using the STATISTICA 6.1 (Stat Soft, Inc. 2002) computer package. The assumptions of normality and homogeneity of variance were confirmed graphically for each data set using boxplots. Egg diameter, moisture, and lipid content of eggs spawned from conditioned and wild caught broodstock during both spawning seasons were compared with a two factor ANOVA with spawning season and conditioned or wild broodstock as fixed factors. Egg sizes and moisture content of unfertilized and fertilized eggs were compared with a *t*-test. *T*-tests were also used to compare individual PUFA and PUFA ratios between eggs from wild and conditioned broodstock. Relationships between the broodstock shell length and weight and egg diameter and batch size, as well as egg characteristics, and the performance of the larvae were explored using simple regression analyses.

RESULTS

Fatty Acid Composition of Broodstock Diets and Feed Intake

Fatty acid composition of abalone diets (seaweeds and formulated feed) is given in Table 1. The formulated feed is lower in

TABLE 1.
Fatty acid composition (%) of different abalone diets used in this study.

Name	Green Alga <i>Ulva australis</i>	Red Alga <i>Champia parvula</i>	Red Alga <i>Gracilaria flagelliformis</i>	Formulated Feed
Saturated fatty acid (SFA)				
C12:0	0.7	1.5	0.0	0.2
C14:0	1.4	7.6	2.9	2.0
C15:0	1.4	1.5	2.9	0.3
C16:0	27.2	30.3	22.9	16.3
C17:0	0.7	0.0	0.0	0.3
C18:0	4.1	1.5	2.9	3.5
C20:0	0.0	0.0	0.0	0.3
Total	35.4	42.4	31.4	22.8
Monounsaturated (MUFA)				
C14:1	0.7	0.0	0.0	0.1
C16:1 (n-9)	3.4	3.0	0.0	2.8
C17:1	0.7	0.0	0.0	0.0
C18:1 (n-9)	6.8	9.1	5.7	13.2
C18:1 (total)	16.3	10.6	5.7	15.8
Total	27.9	22.7	11.4	33.8
Polyunsaturated fatty acid (PUFA)				
C18:2 (n-6)	4.1	0.0	0.0	25.0
C18:3 (n-3)	5.4	0.0	0.0	3.0
C18:3 (n-3)	0.7	0.0	0.0	0.3
C18:4	4.1	0.0	0.0	0.4
C20:2 (n-9)	0.0	0.0	0.0	0.3
C20:4 (n-6)	0.7	4.5	28.6	0.3
C20:5 (n-3)	1.4	3.0	0.0	3.4
C22:5 (n-3)	1.4	0.0	0.0	0.9
C22:6 (n-3)	1.4	0.0	0.0	4.7
Others	17.7	27.3	28.6	5.1
Total	36.7	34.8	57.1	43.4

saturated fatty acids (SFA) than all algal samples. The formulated feed is higher in monounsaturated fatty acids (MFA) than the red algae *Champia parvula* and *Gracilaria flagelliformis* and the green alga *Ulva australis*. The total amounts of PUFA are highest in the red alga *Gracilaria flagelliformis*, followed by the formulated feed and the other algal diets. The relative amounts of PUFAs varied significantly. Formulated feed is high in linoleic acid (18:2n-6) and low in ARA (20:4n-6), whereas both red and green seaweed is low

in linoleic acid. The red seaweeds, especially *Gracilaria flagelliformis*, are high in ARA.

Feed intake of the conditioned broodstock, feeding on a dry formulated feed, was estimated to be $0.10 \pm 0.03\%$ body weight per day. Broodstock feeding on *Ulva* sp. or a mix of red seaweed (*Champia parvula*, *Gracilaria flagelliformis*) were consuming ca. $1.05 \pm 0.08\%$ (\pm SE) or $0.96 \pm 0.08\%$ (\pm SE) of their body weight per day respectively.

Egg Diameter, Batch Size, Proximate Analyses

The egg diameter, number of eggs per batch, weight ($\mu\text{g ind}^{-1}$) and proximate composition of abalone eggs derived from conditioned and wild broodstock are displayed in Table 2. There were no significant differences in average diameter of the eggs from conditioned broodstock ($199.6 \pm 2.5 \mu\text{m}$) and the batches of wild caught broodstock ($199.2 \pm 3.1 \mu\text{m}$), the percentage moisture of the eggs ($90.1 \pm 0.7\%$, $88.6 \pm 0.8\%$ respectively), or the total lipid of the eggs ($231.2 \pm 17.8 \text{ mg g}^{-1}$, $212.8 \pm 20.2 \text{ mg g}^{-1}$ respectively) (Table 3A). There was, however a significant difference between the two spawning seasons in egg diameter and total lipid, but not the moisture of abalone eggs (Table 3A). Eggs were larger during the second season compared with the first spawning season ($204.0 \pm 2.3 \mu\text{m}$, $193.5 \pm 2.4 \mu\text{m}$ respectively, Table 2), but had less total lipid ($178.9 \pm 12.4 \text{ mg g}^{-1}$, $276.8 \pm 10.4 \text{ mg g}^{-1}$ respectively). However, batches were larger during the first spawning season, particularly from conditioned broodstock. There was a significant correlation between broodstock shell length and weight and batch size across the two spawning season (Table 4) indicating that larger broodstock are more fecund. A significant relationship between broodstock shell length or weight and egg diameter was not detected.

Conditioned eggs were significantly heavier with more moisture than eggs derived from wild caught broodstock but had similar amounts of lipid (Table 3B, Table 2). Eggs derived from conditioned and wild broodstock, collected during the second spawning season, were heavier (dry weight) with no significant difference in moisture or lipid (Table 3B, Table 2). The percentage moisture and percentage lipid per egg (% of individual egg weight) were significantly higher during the first spawning season (Table 3C) with lower % lipid per egg in eggs from wild broodstock spawned during the second season (Table 2). Fertilized eggs were 5-10 μm larger than unfertilized eggs ($t = 2.24$, $P = 0.049$) and usually contained more moisture (Table 5, $t = 2.49$, $P = 0.03$).

TABLE 2.

Mean egg diameter (\pm SE), mean number of eggs per batch (\pm SE), mean weight (\pm SE) and mean proximate composition (\pm SE) of *Haliotis laevis* eggs derived from conditioned and wild caught broodstock during two spawning seasons.

	Conditioned Broodstock		Wild Broodstock	
	1. Season ($n = 5$)	2. Season ($n = 4$)	1. Season ($n = 5$)	2. Season ($n = 7$)
Diameter (μm)	198 ± 2.0	202 ± 5.4	189 ± 3.3	206 ± 2.1
No. of eggs per batch ($\times 10^6$)	4.4 ± 1.1	2.1 ± 1.5	5.2 ± 0.5	4.8 ± 1.6
Weight ($\mu\text{g ind}^{-1}$ wet weight)	16.5 ± 2.4	13.4 ± 1.6	8.6 ± 1.0	11.4 ± 1.1
Weight ($\mu\text{g ind}^{-1}$ dry weight)	1.4 ± 0.1	1.6 ± 0.2	0.9 ± 0.1	1.4 ± 0.1
Moisture ($\mu\text{g ind}^{-1}$ wet weight)	15.1 ± 2.4	11.8 ± 1.4	7.7 ± 0.9	10.0 ± 1.1
Moisture (% wet weight)	91.1 ± 1.2	88.0 ± 0.3	89.6 ± 0.7	87.5 ± 1.3
Lipid (% dry weight)	26.6 ± 0.5	18.6 ± 2.9	28.8 ± 2.1	17.1 ± 1.3
Total Lipid (mg g^{-1} dry weight)	266.0 ± 5.1	168.8 ± 26.8	287.5 ± 22.9	170.0 ± 15.9

TABLE 3.

Two factor ANOVAs with spawning season and conditioned versus wild broodstock as fixed factors. A. using data set relating to whole sample and sample weights (mg g⁻¹). B, C. using data set expressed for individual egg weight (µg ind⁻¹). Significant effects (<0.05) are in *italics*.

	Egg Diameter				% Moisture				Total Lipid (mg g ⁻¹)			
	df	MS	F	p	df	MS	F	p	df	MS	F	p
A												
Source												
Conditioned vs wild	1	3.7	0.07	0.797	1	1.7	0.2	0.664	1	2.3	0.002	0.97
Spawning season	1	474.3	8.7	<i>0.009</i>	1	8.90	1.01	0.330	1	43331.1	30.04	<i>0.0001</i>
Cond. vs wild × season	1	166.1	3.05	0.099	1	0.00	0.01	0.950	1	1589.9	1.10	0.31
Error	17	54.5			17	8.8			16	1422.6		
	Egg Weight (µg ind ⁻¹)				Moisture (µg ind ⁻¹)				Lipid (µg ind ⁻¹)			
	df	MS	F	p	df	MS	F	p	df	MS	F	p
B												
Source												
Conditioned vs wild	1	0.67	7.24	<i>0.016</i>	1	107.7	9.1	<i>0.008</i>	1	0.040	3.89	0.066
Spawning season	1	0.67	7.31	<i>0.015</i>	1	1.2	0.1	0.758	1	0.004	0.40	0.539
Cond. vs wild × season	1	0.06	0.59	0.451	1	40.9	3.5	0.080	1	0.001	0.14	0.715
Error	17	13.02			17	11.8			16	0.010		
					% Moisture (ind ⁻¹)				% Lipid (ind ⁻¹)			
	df	MS	F	p	df	MS	F	p	df	MS	F	p
C												
Source												
Conditioned vs wild					1	5.6	0.86	0.367	1	0.49	0.033	0.858
Spawning season					1	33.9	5.18	<i>0.036</i>	1	457.13	30.69	<i>0.0001</i>
Cond. vs wild × season					1	1.3	0.19	0.666	1	15.87	1.066	0.317
Error					17	6.6			16	14.89		

Fatty Acid Analyses

Relative amounts (%) of fatty acids in eggs derived from conditioned and wild caught broodstock are presented in Table 6A and 6B respectively. Saturated fatty acids comprised on average 32% of total fatty acids in conditioned and 34% in eggs of wild broodstock, monounsaturates (MFA) made up 47% of conditioned eggs and 45% of eggs from wild broodstock whereas PUFAs were present only at 12% (5% and 7% for short and long chain PUFA) and 9% (1% and 7% for short and long chain PUFA) in conditioned and wild broodstock eggs respectively (Table 6A, 6B). The ratio of n-3/ n-6 PUFA of 1.5 was significantly lower in eggs of conditioned broodstock than the 2.1 ratio in eggs of wild caught broodstock ($t = -2.6$, $P = 0.02$). Similarly, the ratio of ARA to eicosapentaenoic acid (EPA) was significantly lower in eggs of conditioned broodstock than from wild broodstock (0.4 and 0.8 respectively; $t = -4.68$, $P < 0.001$).

Palmitic acid (16:0) was the dominant saturated fatty acid and

TABLE 4.

Correlation between broodstock shell length and weight and egg diameter and batch size.

	Broodstock Length	Broodstock Weight	Egg Diameter	Number of Eggs per Batch
Broodstock length	1.00	0.95	0.10	<i>0.69</i>
Broodstock weight	<i>0.95</i>	1.00	0.13	<i>0.76</i>
Egg diameter	0.10	0.13	1.00	-0.02
Number of eggs	<i>0.69</i>	0.76	-0.02	1.00

Significant effects are shown in bold and italics ($n = 12$).

oleic acid (18:1) the dominant monounsaturated fatty acid in eggs of both wild caught and conditioned broodstock. The dominant PUFA was EPA (20:5n-3) for both conditioned and wild broodstock derived eggs. ARA (20:4n-6) was present in significantly higher proportion in eggs of wild caught broodstock ($t = -6.24$, $P < 0.001$), linoleic acid (LOA, 18:2n-6), linolenic acid (LNA, 18:3n-3) and docosahexaenoic acids (DHA, 22:6n-3) were significantly more abundant in eggs of conditioned broodstock ($t = 5.99$, $P < 0.001$; $t = 3.11$, $P = 0.006$, $t = 2.58$, $P = 0.02$ respectively). The relative amount of ARA was more than twice as high in wild broodstock, whereas the eggs of conditioned broodstock showed 10 times more of the LOA. Batch 7 was derived from broodstock that had been feeding on formulated feed for a period of 3.5 mo only and showed intermediate levels of both ARA and LOA. Two samples (Batch 15 and 18) were derived from conditioned female broodstock that had been transferred from a formulated diet onto red or green seaweed 3 mo prior to spawning (Table 6A). Eggs from broodstock that had been feeding on *Ulva australis* showed intermediate levels of LOA and similar levels of ARA when compared with the eggs derived from conditioned broodstock. Eggs from red seaweed-fed broodstock showed intermediate levels of

TABLE 5.

Mean diameter (\pm SE; $n = 6$) of unfertilized and fertilized eggs spawned from broodstock *Haliotis laevis*gata.

Eggs	Diameter (µm)	Moisture (%)
Unfertilized	189.2 \pm 2.7	88.9 \pm 1.0
Fertilized	196.8 \pm 2.1	92.2 \pm 1.4

TABLE 6A.

Relative amounts (%) of individual fatty acids of eggs spawned from conditioned *Haliotis laevis* broodstock.

Saturated Fatty Acids	Batch 1	7*	8	9	10	Mean	S.E.	15**	18**
C12:0	0.3	0.4	0.3	0.4	0.3	0.4	0.0	0.4	0.3
C14:0	6.0	7.5	5.6	6.6	5.7	6.3	0.4	7.3	4.7
C15:0	0.2	0.3	0.2	0.2	0.2	0.2	0.0	0.4	0.1
C16:0	25.9	25.6	25.5	26.1	24.8	25.6	0.2	22.5	21.8
C17:0	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.4	0.2
C18:0	2.9	1.8	2.7	2.4	2.6	2.4	0.2	1.7	2.3
Total	35.5	35.8	34.5	35.8	33.8	35.1	0.4	32.4	29.5
Monounsaturated fatty acids									
C14:1	0.4	0.7	0.4	0.5	0.4	0.5	0.1	0.6	0.3
C16:1 (n-9)	5.6	7.4	5.1	6.4	5.6	6.0	0.4	5.9	4.1
C16:1 (total)	6.6	8.6	5.9	7.4	6.5	7.0	0.5	7.1	4.9
C18:1 (n-9)	17.8	15.7	18.1	17.9	17.7	17.4	0.4	12.0	15.9
C18:1 (n-7)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14.4	13.1
C18:1 (total)	34.2	35.0	34.0	35.0	34.0	34.5	0.2	26.7	29.3
C20:1 (n-9)	1.4	1.3	1.5	1.2	1.4	1.4	0.1	1.2	1.5
C20:1 (total)	5.4	4.8	5.7	4.7	5.5	5.2	0.2	4.2	5.1
Total	46.6	49.2	46.0	47.6	46.6	47.2	0.6	38.6	39.6
Polyunsaturated fatty acids									
C16:2 (total)	1.5	1.4	1.8	1.4	1.8	1.6	0.1	1.3	1.3
C16:4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2	0.3
C18:2 (n-6) LOA	2.9	1.1	3.0	2.7	2.8	2.5	0.4	0.5	1.9
C18:3 (n-6)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2	0.1
C18:3 (n-3) LNA	0.6	0.2	0.5	0.7	0.6	0.5	0.1	0.1	0.0
C20:2 (n-9)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.4	1.6
C20:2 (total)	2.1	0.6	1.9	1.8	2.2	1.7	0.3	0.7	1.9
C20:4 (n-6) ARA	0.7	1.6	1.0	0.6	0.8	0.9	0.2	1.9	0.5
C20:5 (n-3) EPA	2.0	2.3	2.0	1.7	2.3	2.1	0.1	2.3	1.9
C22:5 (n-3)	1.4	2.3	1.6	1.3	1.6	1.7	0.2	1.9	1.3
C22:6 (n-3) DHA	0.7	0.2	0.8	0.7	0.9	0.6	0.1	0.0	0.7
Others	6.1	5.4	6.7	5.6	6.7	6.1	0.3	4.9	7.0
Total	11.8	9.7	12.7	10.9	12.9	11.6	0.6	9.6	11.4

* Batch 7 derived from broodstock, which was only conditioned for a period of 3.5 months.

** Batch 15 and 18 derived from broodstock, which were conditioned on formulated feed but fed seaweed for 3 months prior to spawnings: batch 15 fed red seaweeds, batch 18 fed *Ulva* sp.

LOA and levels of ARA similar to eggs derived from wild caught broodstock.

Offspring Performance

The results of larval and postlarval performance of seven selected batches are shown in Table 7. The hatch rate was usually higher during the first spawning season, the best larval survival and overall survival was obtained from offspring derived from one female broodstock that was conditioned on formulated feed but was fed red seaweed for 3 mo prior to spawning during the second spawning season (Batch 15).

Correlation analyses were undertaken for all combinations of egg characteristics and the performance of the larvae (Table 8). There were strong and significant negative correlations between egg diameter and total lipid content ($r = -0.89$), between egg diameter and hatch rate ($r = -0.93$), and a positive correlation between total lipid content of the eggs and hatch rate. There was a strong negative correlation between the percentage moisture of the eggs and the performance at settlement ($r = -0.95$). There was also a positive correlation between the survival of the larvae during larval rearing and the survival of the postlarvae 2 wk after settlement ($r = 0.99$).

DISCUSSION

This study showed high variability in diameter, weight, and biochemical composition of abalone eggs between spawning seasons. In addition, the total number of eggs per batch declined during the second spawning season particularly in batches spawned from conditioned broodstock. However, broodstock spawned during the second season were slightly smaller. Overall animals were spawned on average 1 mo earlier during the second spawning season compared with the animals spawned during the first spawning season. Both could have contributed to the smaller batch sizes during the second season and the observed differences in moisture and lipid between the two spawning seasons. Other studies have established that temperature and conditioning intervals influence egg production of greenlip and blacklip abalone (Plant 2002, Grubert & Ritar 2003). Shepherd et al. (1992) demonstrated a relationship between broodstock size and fecundity of six wild populations of *Haliotis laevis*. In this study we found a positive correlation between broodstock size and weight and the batch size of spawned eggs.

Compared with the proximate composition of *Haliotis rubra* eggs from the wild (Litaay et al. 2001), *Haliotis laevis* eggs in this study were about two to three times heavier containing 10% to

TABLE 6B.

Relative amounts (%) of individual fatty acids of eggs spawned from wild caught *Haliotis laevis* broodstock.

Saturated Fatty Acids	Batch 2	3	4	5	6	11	12	13	14	16	19	20	Mean	S.E.
C12:0	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0
C14:0	7.4	6.9	6.8	7.3	6.4	7.0	7.1	6.7	6.6	7.0	6.1	5.6	6.7	0.5
C15:0	0.5	0.3	0.3	0.4	0.4	0.4	0.3	0.3	0.4	0.3	0.3	0.4	0.4	0.0
C16:0	26.2	26.4	25.8	28.0	26.6	23.8	22.9	22.2	21.4	21.1	22.7	23.6	24.2	2.3
C17:0	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0
C18:0	1.9	2.1	2.1	2.2	2.2	1.5	1.5	1.5	1.3	1.2	1.8	2.1	1.8	0.4
Total	36.6	36.4	35.7	38.5	36.2	33.3	32.5	31.3	30.2	30.2	31.5	32.2	33.7	2.8
Monounsaturated fatty acids														
C14:1	0.7	0.5	0.6	0.6	0.5	0.7	0.7	0.6	0.7	0.9	0.5	0.4	0.6	0.1
C16:1 (n-9)	8.5	6.8	7.7	7.1	7.0	7.6	7.0	6.3	7.2	8.0	5.8	5.2	7.0	0.9
C16:1 (total)	9.7	7.9	8.8	8.2	7.9	8.8	8.1	7.2	8.4	9.2	6.7	6.0	8.1	1.0
C18:1 (n-9)	15.6	17.7	18.1	16.7	18.4	12.8	12.2	11.2	12.9	11.1	14.8	16.4	14.8	2.7
C18:1 (n-7)	n.d.	n.d.	n.d.	n.d.	n.d.	15.3	16.1	12.8	16.7	15.4	15.1	14.7	15.2	1.2
C18:1 (total)	33.8	36.4	37.4	34.5	36.8	28.4	28.7	24.3	29.8	26.9	30.1	31.4	31.5	4.2
C20:1 (n-9)	1.1	1.4	1.3	1.1	1.4	0.8	0.9	0.8	0.9	0.7	1.3	1.3	1.1	0.3
C20:1 (total)	4.7	5.4	4.9	5.1	5.3	3.9	4.1	3.5	3.8	3.5	4.2	4.6	4.4	0.7
Total	48.9	50.3	51.7	48.3	50.6	41.7	41.6	35.7	42.8	40.5	41.5	42.4	44.7	5.1
Polyunsaturated fatty acids														
C16:2 (total)	1.2	1.3	1.3	1.3	1.3	1.2	1.3	1.1	1.2	1.2	1.3	1.2	1.2	0.1
C16:4	n.d.	n.d.	n.d.	n.d.	n.d.	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.2	0.0
C18:2 (n-6) LOA	0.2	0.2	0.3	0.2	0.3	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.2	0.0
C18:3 (n-6)	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.0
C18:3 (n-3) LNA	0.2	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
C20:2 (n-9)	n.d.	n.d.	n.d.	n.d.	n.d.	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.0
C20:2 (total)	0.2	0.2	0.3	0.3	0.2	0.5	0.6	0.4	0.6	0.6	0.5	0.5	0.4	0.2
C20:4 (n-6) ARA	1.9	2.0	1.8	1.9	1.9	2.0	2.3	1.9	2.1	2.0	1.8	1.7	1.9	0.2
C20:5 (n-3) EPA	3.6	2.8	2.5	2.7	2.8	2.3	2.4	2.3	2.1	2.4	2.4	2.4	2.6	0.4
C22:5 (n-3)	2.7	2.2	1.5	2.1	2.0	2.0	1.8	1.8	1.6	1.9	2.0	1.9	2.0	0.3
C22:6 (N-3) DHA	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Others	4.5	4.7	4.7	4.7	4.7	3.8	3.9	3.2	4.0	3.8	4.1	4.1	4.2	0.5
Total	10.0	8.7	7.8	8.5	8.5	8.7	9.2	8.0	8.2	8.6	8.7	8.5	8.6	0.6

15% more moisture and ca. 10% more lipid per egg during the first spawning season but similar amounts during the second spawning season. The difference in moisture between eggs of these studies might be partially explained by differences in time elapsed be-

tween spawning and measuring of the eggs (e.g., eggs might have been more hydrated). In this study, special care was taken that samples were measured approximately at the same time and no fixative was used. However, differences in total lipid cannot have been influenced by different sampling techniques. Total lipid expressed in mg g^{-1} was not given in the study by Litaay et al. (2001). Total lipid (mg g^{-1}) of digestive gland tissue of the abalone *Haliotis fulgens* (Nelson et al. 2002) was, however, much lower than total lipid in eggs of the abalone *H. laevis* determined in this study. Nelson et al. (2002) determined the lipid content of

TABLE 7.

Hatch rate (%) and larval survival to settlement (%), performance at settlement and post-larval survival 2 weeks after settlement of selected batches derived from conditioned (c) and wild (w) caught broodstock during the two spawning seasons.

Season	Treat-ment	Batch	Hatch Rate (%)	Larval Survival (%)	Settlement (%)	Post-larval Survival (%)
1	c	1	95	15	—	—
		7*	95	15	—	—
	w	2	100	25	61	0.3
		3	99	30	36	29
2		6	4	29	<30	—
	c	15**	77	100	64	69
	w	20	80	16	76	7

* Batch 7 derived from broodstock that was only conditioned for a period of 3.5 months.

** Batch 15 derived from broodstock that were conditioned on formulated feed but fed red seaweed for 3 months prior to spawning.

— Larvae were not settled.

TABLE 8.

Correlation between egg characteristics and larval performance.

	Egg Diameter	Moisture (%)	Lipid (%)	Hatch (%)	Larval Survival	Set (%)
Egg diameter		-0.36	-0.89	-0.93	0.48	0.35
Moisture	-0.36		0.74	0.66	-0.47	-0.95
Lipid (%)	-0.89	0.74		0.98	-0.50	-0.72
Hatch (%)	-0.93	0.66	0.98		-0.63	-0.64
Larval survival (%)	0.48	-0.47	-0.50	-0.63		0.30
Set (%)	0.35	-0.95	-0.72	-0.64	0.30	
Post-larval survival (%)	0.60	-0.51	-0.62	-0.74	0.99	0.37

Significant effects are shown in bold and italics ($n = 7$).

gonad tissue over a 1-y period and showed variations between 18 and 101 mg g⁻¹. In this study we collected eggs during two concurrent natural spawning seasons of *H. laevigata* and noticed significant differences between the two spawning seasons in both eggs derived from conditioned and wild broodstock with total lipid as low as 118 mg g⁻¹ (1st spawning season) and as high as 350 mg g⁻¹ (2nd spawning season). Egg samples were taken earlier in the season during the second spawning season, the sampling started in early October and finished in December with most of the samples collected during October to November 2002. Samples during the first spawning season were mainly taken during December 2001 and January 2002. It is conceivable that eggs were not quite as mature during the second spawning season and thus smaller than during the first spawning season. Further studies are needed to establish the relationship between egg size, biochemical composition, and maturity of abalone eggs.

Nelson et al. (2002) demonstrated that C₂₀ PUFA in the digestive gland can vary between seasons and that variations over the year coincided with variation in their macroalgal diet. Similarly, Dunstan et al. (1996) found elevated levels of short chain linoleic acid (LOA) and reduced levels of ARA (ARA, 20:4n-6) in juvenile abalone feeding on formulated feeds, despite the fact that the formulated feed contained high proportions of the ARA precursor LOA (18:2n-6). Australian species of abalone have a preference for red seaweed such as *Gracilaria* spp. (Shepherd & Steinberg 1992), which are rich in the PUFAs, ARA, and EPA (Mai et al. 1996, Dunstan et al. 2000, Table 1). A formulated feed used in Australia, however, is low in ARA (Dunstan et al. 1996, Table 1). Results of the present study indicate that the PUFA composition of the broodstock diets influenced the PUFA profile in abalone eggs. Eggs derived from wild caught broodstock were higher in ARA, the formulated feed used is high in LOA (Table 1), and eggs from broodstock feeding on this formulated feed showed a much higher level of this PUFA. In accordance with our findings, Soudant et al. (1996) showed that the PUFA composition of the diets influenced the PUFA profiles in eggs of the great scallop. Results of our study also indicate that 3 mo of feeding on an altered diet is sufficient to alter the fatty acid profile of the eggs. Notably the progeny from one particular batch of formulated diet fed conditioned broodstock that was transferred onto a red seaweed diet showed the best performance.

Arachidonic acid is essential for marine fish (Sargent et al. 1999), and until recently the importance of this fatty acid had been largely overlooked (Bell & Sargent 2003). Whether ARA is essential to abalone needs to be examined (Dunstan et al. 1996). However, Dunstan et al. (2002) demonstrated that most of the final tissue ARA originated from dietary linoleic acid (LOA) and, because formulated feed contains large amounts of this fatty acid, they concluded that it is unlikely that ARA is limiting in abalone. Although high levels of ARA may not be required for muscle growth, it may be required for ovogenesis and embryogenesis. ARA is a major precursor of prostaglandins, which influence reproduction in molluscs (Osada et al. 1989). There is growing evidence that ARA is important in reproduction of marine fish (Bell & Sargent 2003) and Soudant et al. (1996) demonstrated that DHA, EPA, and ARA were preferentially incorporated into the polar lipid fraction of scallop eggs. They suggested that the higher levels of ARA in eggs than in larvae of the scallop indicated its specific role during ovogenesis. Recently, Nelson et al. (2002) showed that ARA was markedly lower in the digestive gland than in the foot tissue of the abalone *Haliotis fulgens* and concluded that this fatty acid may have been converted to prostaglandins, which

indicates its function in gametogenesis. In accordance with our findings, Dunstan et al. (1996) reported that ARA was present at 0.4% in formulated diets and at 1.1% in the macroalga *Ulva australis*. These results together with our findings indicate that ARA was preferentially incorporated into the eggs of broodstock feeding on formulated feed and *Ulva australis*. In our study, the levels of ARA were higher in eggs than in the respective diets of the broodstock, except of the *Gracilaria* diet. In addition, several authors have indicated that beyond this, certain fatty acids play a vital role in determining the success of metamorphosis in bivalves (Helm et al. 1973, Kraeuter et al. 1982, Wilson et al. 1986). Abalone have lecithotrophic larvae, which cannot digest particles but are able to take up nutrients from seawater (Jaekle & Manahan 1989). Consequently the lipid of the eggs must fuel major parts of the energetics of larval metamorphosis. Jensen et al. (1990) showed that the metamorphosis of *H. rufescens* larvae was influenced by free fatty acids, especially ARA. A very low relative amount of PUFAs compared with SFA and MUFA was found in the abalone eggs. On average, PUFAs contributed only 10% of the total fatty acid pool. In contrast, Soudant et al. (1996) demonstrated that up to 50% of the fatty acids were PUFAs in scallop eggs. PUFAs in abalone diets (algal diets and formulated feed) on the other hand, contribute about 35% to 56% but this was not reflected in the eggs. Nelson et al. (2002) showed high variability in relative amounts of PUFAs in digestive gland tissue of *H. fulgens* between seasons and diets, ranging between 7% and 42%. In this study, however, the relative proportions of saturated, monounsaturated, and PUFAs in eggs of the abalone *H. laevigata* were very consistent in all samples regardless of the broodstock diet probably because of the metabolic necessity to maintain equilibrium between these fatty acid groups. It is conceivable that SFA and MUFA are stored in abalone eggs and used as an energy source for the developing larvae.

Correlation analyses indicated that smaller eggs had higher lipid content and that larvae from these batches hatched better; larvae hatching from eggs with lower moisture content performed better at settlement.

The results of this study demonstrate high variability in proximate biochemical composition and fatty acid profiles of abalone eggs between batches derived from conditioned and wild broodstock as well as between two consecutive spawning seasons. Our preliminary results provide initial evidence that biochemical composition of abalone eggs are influenced by the broodstock diets and these in turn may affect the performance of the progenies. The results related to offspring performance have to be treated carefully because only seven batches were compared during the two spawning seasons. Further work is planned to provide more information on egg provisioning and offspring performance in relation to the composition of the broodstock diets, particularly in relation to fatty acid content. If abalone aquaculture is to become more advanced and sustainable, broodstock have to be successfully conditioned on farms to secure high quality eggs and larvae, because this will enable selective breeding programs for a further development of the industry.

ACKNOWLEDGMENTS

The authors thank the staff for their help and support during spawnings and Dr Greg Maguire, Dr Brett Glencross, and Dr Sagiv Kolkovski for their helpful comments. Great Southern Marine Hatcheries in Albany, Western Australia hosted this experiment.

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SETTLEMENT OF ABALONE (*HALIOTIS IRIS*) LARVAE IN RESPONSE TO FIVE SPECIES OF CORALLINE ALGAE

RODNEY D. ROBERTS,^{1,*} HEINRICH F. KASPAR¹ AND RICHARD J. BARKER³

¹Cawthron Institute, Private Bag 2, Nelson, New Zealand; ²Department of Marine Science, University of Otago, P.O. Box 56, Dunedin, New Zealand; ³Department of Mathematics and Statistics, University of Otago, P.O. Box 56, Dunedin, New Zealand

ABSTRACT Abalone recruits are found predominantly on crustose coralline algae (CCA) and recruitment seems to vary among coralline species or growth-forms. Spatial patterns in the recruitment of marine invertebrates can be formed by processes acting before, during, or after settlement. This study used laboratory experiments to examine the settlement of *Haliotis iris* larvae on five species of CCA. When the CCA species were presented individually to larvae, all induced >88% of larvae to attach within 1 day, and >80% to metamorphose within 3 days. When given a choice of the five CCA, larvae settled most on *Pneophyllum coronatum* and *Hydrolithon rupestris*, and settled least on *Mesophyllum printzianum*. *Phymatolithon repandum*, and *Lithophyllum carpophylli* gave intermediate results. The speed of metamorphosis in the no-choice experiments mirrored the species preferences in the choice experiments, with metamorphosis occurring most rapidly on *Pneophyllum coronatum* and *Hydrolithon rupestris*. The two preferred species were thin crusts, whereas the two least preferred species formed thick, morphologically complex crusts. In a choice experiment, the proportion of larvae choosing a CCA specimen showed no correlation with bacterial density ($r = -0.25$, $P = 0.52$, $n = 25$), but a weak positive correlation with diatom density ($r = 0.52$, $P = 0.02$, $n = 25$). However, diatom densities were low on all CCA species, and are unlikely to be a primary cause of settlement preference. It is hypothesized that at least some of the variation in recruitment among CCA species or growth forms observed in the wild is determined by selective settlement.

KEY WORDS: abalone, biofilm, coralline algae, larval settlement, metamorphosis, recruitment.

INTRODUCTION

Patterns of recruitment in marine larvae may be caused by processes occurring before, during, or after settlement (Connell 1985). The relative importance of these stages in determining recruitment varies among species and locations (e.g., Keough & Downes 1982, Connell 1985, Gaines & Roughgarden 1985, Woodin 1986, Rowley 1989, McShane 1992, Stoner et al. 1996, Walters & Wethey 1996, Mundy & Babcock 2000).

Abalone recruits are found almost exclusively on crustose coralline algae (CCA) (Shepherd & Turner 1985, McShane & Smith 1988, Day & Branch 2000). Based on this selection of a particular habitat it was initially suggested that differential postsettlement mortality is unlikely to be important in determining abalone recruitment (McShane 1991, McShane 1992). However, the broad grouping "crustose coralline algae" includes a wide range of species and growth forms (Woelkerling et al. 1993). Recent research on abalone ecology has recognized this complexity and demonstrated that the species and growth form of CCA can influence the number of abalone recruits (McShane 1996, Shepherd & Daume 1996, Daume et al. 1999, Day & Branch 2000). In South Australia, "lumpy" and "uneven encrusting" growth forms had more recruits of *Haliotis laevis* and *Haliotis scalaris* than other coralline growth forms (Shepherd & Daume 1996) and in South Africa, "knobbly" corallines contained the highest densities of *Haliotis midae* recruits (Day & Branch 2000).

Such patterns could be caused by selective settlement or differential postsettlement survival. Work to date has focused on the latter. It has been suggested that morphologically complex CCA growth forms may be more suitable than flat crusts because they provide greater protection of postlarvae from wave shear (postulated by McShane & Naylor 1995 and McShane 1996, but not supported by experimental evidence of Naylor & McShane 1997a) inadvertent ingestion (Fletcher 1987, McShane 1991), predatory

fish (McShane 1996) or because they protect pockets of microbial food from access by larger grazers (Shepherd & Daume 1996, Day & Branch 2000). On the other hand, topographically complex surfaces may increase the thickness of the diffusive boundary layer by impairing water movement near the substrate, resulting in more extreme fluctuations in water chemistry in the postlarval environment (Kaspar 1992, Day & Branch 2000). Morphologically complex CCA growth forms generally grow as thick crusts. These harbor predatory worms that can decimate populations of newly settled abalone (Morse et al. 1979, Naylor & McShane 1997a, Naylor & McShane 1997b).

Coralline algae are among the most effective larval settlement cues for all species of abalone tested to date (Roberts 2001a). Other natural surfaces such as biofilms can induce larval metamorphosis, but few approach the speed and consistency of CCA (Roberts 2001a, Roberts 2001b). Little work has been done on the extent to which settling abalone larvae discriminate among coralline species or growth forms.

Discrimination among substrata is well demonstrated for settling marine invertebrate larvae (reviewed by Crisp 1974, Pawlik 1992, Rodriguez et al. 1993). Some studies have demonstrated settlement preference in multiple choice experiments (e.g., Kirchner et al. 1982, Miron et al. 1996, Stoner et al. 1996, Wicczorek & Todd 1997, Snelgrove et al. 1998, Daume et al. 1999). These choice experiments test the relative preference of larvae for a particular substrate when faced with a range of options, whereas experiments that present cues individually test the absolute inducing ability of a substrate for larvae that are given no other option. The former is more relevant to the situation faced by a larva in nature.

The few data available for abalone larvae provide conflicting evidence on selectivity among types of CCA. Morse et al. (1980) and Morse and Morse (1984) reported equally strong settlement in response to various species of CCA, but the species were presented individually rather than as multiple choices. In contrast, larvae of *Haliotis laevis* metamorphosed in greater numbers on the thick,

*Corresponding author. E-mail: rodney.roberts@cawthron.org.nz

lumpy crusts of *Sporolithon durum* than on the encrusting *Hydrolithon rupestre* (= *Hydrolithon rupestris*) or the warty *Mesophyllum engelhartii* (Daume et al. 1999). Larvae also metamorphosed more quickly on *Sporolithon* with a lumpy growth form than the same species with an encrusting growth form. Both patterns were consistent in choice and no-choice experiments.

Data comparing the performance of types of CCA for the settlement of invertebrates other than abalone are also sparse. The scleractinian coral *Agaricia agaricites humilis* metamorphosed strongly in response to only 3 of 6 CCA species presented individually (Morse et al. 1988). The soft coral *Acyonium siderium* metamorphosed in response to both of two CCA species (Sebens 1983).

More data are required to assess the importance of settlement preferences in determining distributions of recruits among types of CCA. The ecological justification for such studies is compelling. CCA are abundant from tropical to polar waters (Johansen 1981, Steneck 1986) occupying more hard substratum within the photic zone than any other organism (Steneck & Paine 1986). CCA trigger the settlement of numerous species of marine invertebrate larvae (Pawlik 1992, Johnson et al. 1991) and difficulties with the taxonomy and identification of CCA have obscured the species specificity of many interactions involving CCA (Steneck & Paine 1986).

The present study compares abalone larval settlement on 5 CCA species with diverse growth forms, in choice and no-choice experiments. It was found that all CCA species induced metamorphosis if presented individually, but that larvae exhibited preferences when given a choice.

METHODS

Larval Rearing and Bioassays

Ripe adult *Haliotis iris* Gmelin 1791 were spawned by the hydrogen peroxide technique (Morse et al. 1977) and the larvae reared in flowing seawater as described previously (Roberts & Nicholson 1997) until taken for assays. Competent larvae were added to the wells of tissue culture plates (Falcon 3043 or 3047) containing CCA in 2 mL (no choice experiments) or 3.3 mL (choice experiments) of 0.2 μ m-filtered seawater with 150 μ g/mL of both penicillin G sodium (Sigma) and streptomycin sulphate

(Sigma). Assay plates were wrapped in foil and incubated at $17 \pm 0.5^\circ\text{C}$. To quantify settlement, the whole of each CCA fragment and its well were examined microscopically (Roberts & Nicholson 1997) after ~1 and 2.5 to 3 days of incubation. Counts were converted to percentage of larvae showing: (a) "shell growth" (velum shed and peristomal growth visible); (b) "metamorphosis" (velum shed, with or without shell growth); and (c) "attached" (metamorphosed or attached by foot). "Settlement" is used as a general term encompassing larval attachment and metamorphosis (Roberts 2001a). Negative controls of filtered seawater (with clay in Experiment 3) showed 0% to 0.3% metamorphosis within 3 days (data not presented). Experimental details are presented in Table 1.

Collection and Preparation of CCA

Details and taxonomic authorities of the five CCA species used are given in Table 2. Fragments of ~0.15 to 0.3 cm² "floor area" (defined later) were prepared by cutting the plastic on which they were growing (for the fragile *Pneophyllum coronatum* and *Hydrolithon rupestris*) or breaking off fragments of the plant with a knife (*Lithophyllum carpophylli* and *Mesophyllum printzianum*) or a hammer and chisel (*Phymatolithon repandum*). Each assayed fragment was from a separate plant, and each plant of *Lithophyllum carpophylli* was from a separate host plant of *Carpophyllum maschalocarpum*.

In Experiments 1 and 3 the fragments of CCA were held in filtered seawater for <30 min before being moved into bioassay chambers. In Experiment 2, fragments were soaked in ~1L of static 1- μ m filtered seawater for 4 days at 17°C before bioassay, with the intention of removing dissolved components leaking from broken edges of the CCA. This holding period without grazers would also alter the biofilm on the CCA (not quantified). In Experiment 3, the floor of each bioassay chamber was filled with a flattened plug of "colour clay" (Trident Toy Co. Ltd, Hutton, Avon, England) to ~2.5 mm depth and the CCA fragments embedded in the clay so that their upper (live) surface was flush with the surface of the clay. The clay was molded to fit closely the edges of the samples. The intention of this treatment was to make all CCA species equally accessible to crawling larvae (see also Discussion).

For choice experiments, the five CCA fragments in a replicate were randomly placed in a circle on a radius two-thirds that of the

TABLE 1.

Details of experiments conducted during this study. Start date is the day on which larvae were added to CCA. Larval stage was determined by the number of fully chitinized transverse rows of teeth on the larval radula (Tong and Moss 1992).

Experiment Number	Larval Batch; and Start Date	Larval Stage (Rows of Radula Teeth)	Number of Replicates	No. Larvae Per Well Mean \pm SE (n)	Purpose
Choice Exp. 1	1; 18/9/98	8-9	6	192 \pm 30 (6)	Determine relative settlement of larvae given a choice of five CCA species.
Choice Exp. 2	1; 22/9/98	12-13	6	211 \pm 27 (6)	Repeat Choice Exp. 1 but with CCA fragments soaked for 4 days to reduce effects from inducers leaking from damaged plants.
Choice Exp. 3	3; 21/4/99	7-8	6	92 \pm 8 (6)	Repeat of Choice Exp. 1 but with a separate batch of larvae and with CCA fragments embedded in clay to standardize their accessibility to crawling larvae.
No-choice Exp. 1	1; 18/9/98	8-9	5	58 \pm 5 (30)	Quantify settlement of larvae in response to the 5 CCA species presented individually for comparison with Choice Exp. 1.
No-choice Exp. 3	3; 21/4/99	7-8	6	43 \pm 5 (30)	As previous for comparison with Choice Exp. 3.

TABLE 2.

Morphologic and ecological features of the crustose coralline algae (CCA) used in this study.

CCA Species	Source	Morphology	Crust Thickness	Likely Grazers
<i>Pneophyllum coronatum</i> (Rosanoff) Penrose in Chamberlain	On plastic plates in GACL hatchery	Encrusting	~0.2 mm	Juvenile <i>Haliotis iris</i> and small crustacea
<i>Hydrolithon rupestre</i> (Foslie) Penrose	On plastic plates in GACL hatchery	Encrusting	~0.2 mm	Juvenile <i>Haliotis iris</i> and small crustacea
<i>Phymatolithon repandum</i> (Foslie) Wilks and Woelkerling	On stones from shallow subtidal at Cable Bay (173°24'E, 41°11'S)	Encrusting to warty	~0.5 mm	Mixture of grazing species but not abalone
<i>Lithophyllum carpophylli</i> (Heydrich) Heydrich	On stipes of sublittoral fringe <i>Carpophyllum maschalocarpum</i> (Phaeophyceae) on shore by GACL	Foliose	2–4 mm	Small gastropods and crustaceans but not abalone
<i>Mesophyllum printzianum</i> Woelkerling and Harvey	On stable boulders in sublittoral fringe on shore by GACL	Warty to fruticose	2–5 mm	Mixture of grazers including <i>Haliotis iris</i>

GACL = Glenhaven Aquaculture Centre Ltd (174°21.2'E, 41°11.4'S). Morphology terminology follows Woelkerling et al. (1993).

tissue culture plate well (Falcon 3043), with the center of the well unoccupied. For no-choice experiments the CCA fragment was placed in the center of the tissue culture plate well (Falcon 3047) with species randomly arranged in the plates.

Phymatolithon repandum and *Mesophyllum printzianum* have uniporate gametangial conceptacles and multiporate tetrasporangial conceptacles, making these life cycle stages easy to distinguish. A previous experiment showed that both life cycle stages induced equally strong larval attachment and metamorphosis within 60 h (data not shown), so life cycle stage of CCA was excluded from further consideration.

For Choice Experiment 1, fragment size was estimated by measuring length and width of fragments with an eye-piece micrometer in a binocular microscope. In Experiment 3, the size of CCA fragments was determined by image analysis (Optimas 6) of video-records. Fragment size was measured as the area of the CCA when viewed from above (= "floor area"), taking no account of the greater surface area of the topographically complex corallines (notably *Mesophyllum printzianum*). The floor area measurement was regarded as the most appropriate measure of "target size" (see Discussion).

Scanning Electron Microscope Analysis of Biofilms

Samples of coralline algae were prepared for scanning electron microscope (SEM) analysis by 2 methods. A simple preparation procedure (termed "SEM method 1") was designed to minimize loss of the microbial population on the CCA surface. A more complex procedure (termed "SEM method 2") was followed to preserve the form of microbes and other fine structures on the surface of the corallines.

SEM method 1: for each replicate in Experiment 3, a sample from the same plant was preserved in 2% glutaraldehyde (Merck) then air dried in a laminar flow cabinet. Dried samples were attached to aluminum stubs with double-sided carbon tape and silver paint then sputter-coated with gold/palladium before viewing with a Cambridge S360 Stereoscan SEM. On each sample, bacteria were counted in 12 × 5000 magnification fields of view (each 24 μm × 17 μm) and diatoms in 12 × 1000 fields of view (each 121 μm × 82 μm). Samples were scanned systematically, and nonoverlapping fields of view were haphazardly located

along the scan path. Additional samples of each CCA species, collected from the same locations, were fixed in 4% formalin in seawater, air dried, sputter coated, and viewed with a Hitachi 5-2250N SEM. Diatoms were counted in 12 × 1000 fields of view (each 127 × 98 μm).

SEM method 2: Samples of each species of CCA from the same sources were placed directly into foil-wrapped containers with 2.5% glutaraldehyde in the field. A day later samples were transferred to 1% osmium tetroxide for 24 h, then desalinated with a graded series of 0, 25, 50, 75, and 100% distilled water in filtered seawater (15 min per step), dehydrated with a graded series of 10, 30, 50, 70, 90, 95, and 100% ethanol in distilled water (15 min per step), and critical point dried (Hayat 1989, Johnson et al. 1991). Samples were coated and scanned as earlier.

Statistical Analyses

Correlations were tested among planned comparisons using the Dunn-Sidak method to maintain the experiment-wise Type I error rate at 0.05 (SPSS Inc. 1999). Diatom and bacterial densities used in correlations related only to the surface of the CCA fragments, ignoring the surrounding surfaces. In data from the choice experiments, the ANOVA assumption of independent errors was violated—knowing that a larva settled on CCA species A also tells us that it did not settle on the other species, so the errors are correlated. Settlement preferences across the three replicated choice experiments were analyzed by fitting a log-linear model to the 3-way classification of CCA species (5 levels), experiments (3 levels), and replicates within experiments (6 levels). Only those animals that settled on CCA were used in the analysis, and it was assumed that the effects of day and replicate on settlement preference were additive rather than interactive (i.e., the 3 way interaction term was omitted from the model). The analysis used a quasi-likelihood approximation to an over-dispersed log-linear model, with random experiment and replicate effects (Link 1999). This approach treats the random effects as fixed effects, and assumes that larvae behave independently. This leads to an intentionally over-dispersed model, where the over-dispersion is caused by experiment and replicate effects and/or by nonindependent behavior among larvae. There is a corresponding intentional underestimation of the sampling variance and test statistics, which are

then adjusted using the quasi-likelihood adjustment factor (= ratio of goodness of fit statistic to its degrees of freedom) (Link 1999). Lack of a significant replicate effect after adjustment for over-dispersion (see Results) allowed the data to be reduced to a 2-way classification (5 CCA species, 3 experiments). To obtain selection probabilities for each CCA species, the data from the 3 experiments were combined by a weighted average that took into account the variance within each experiment (Burnham et al. 1987). The reported 95% confidence intervals include variance components from the estimation of the selection probabilities within experiments, and the between-experiment variation.

RESULTS

Experiments With a Choice of Coralline Species

In Choice Experiment 1, *Hydrolithon rupestris* and *Pneophyllum coronatum* attracted more than twice as many settling larvae as the other three CCA species (Fig. 1A). Few larvae (0.7% of total) settled on *Mesophyllum prinzianum*. Almost all of the larvae had metamorphosed, and most had begun to grow postlarval shell, within 22 h. Nearly half of the larvae were found on the floor or walls of the assay chamber, rather than on the CCA themselves (Fig. 1A). Fragment size was almost identical in four of the CCA species (averages of 0.194 to 0.197 ± 0.02 cm²) whereas the most preferred species for settlement, *Hydrolithon rupestris*, had slightly smaller fragments (0.15 ± 0.03 cm²).

In Choice Experiment 2 the order of preference for the CCA species was almost the same as in Choice Experiment 1 except that *Hydrolithon rupestris* was less preferred, slipping to second choice and comparable to *Phymatolithon repandum* (Fig. 1B). All larvae on CCA had metamorphosed, but of the 24% not on CCA, 8% had attached but not metamorphosed, and 7% had not yet attached (Fig. 1B). The proportion of larvae not on CCA was about half of that in Choice Experiment 1 (Fig. 1A).

In Choice Experiment 3, the order of preference for CCA species was again similar with *Pneophyllum coronatum* and *Hydrolithon rupestris* clearly preferred (Fig. 1C, D). *Phymatolithon repandum* was less preferred in this experiment than previously. Larvae were slower to metamorphose in this experiment, with 46% of larvae attached (mostly to CCA fragments) but not metamorphosed after 23 h. However, the majority of abalone found on the two preferred species, *Pneophyllum coronatum* and *Hydrolithon rupestris*, had metamorphosed within 23 h. The size of CCA fragments varied little among the CCA species (Fig. 2C), and showed no correlation with the proportion of settlers ($r = 0.02$, $P = 0.999$, $n = 30$).

Analysis of the data from the three choice experiments demonstrated a strong difference in settlement probabilities among CCA species ($F_{12, 40} = 17.55$, $P < 0.0001$). *Pneophyllum coronatum* and *Hydrolithon rupestris* attracted significantly more settlers than the other CCA species (Fig. 3). The goodness-of-fit statistic from the model omitting the 3-way interaction term provided evidence of over-dispersion ($\chi^2_{40} = 174.27$, $p < 0.001$) indicating that either substrate settlement probabilities varied among experiments and/or replicates, or that larvae were not behaving independently. After accounting for over-dispersion, there was little evidence of variation between replicates ($F_{20, 40} = 1.192$, $p = 0.31$) but strong evidence of variation in settlement probabilities between days ($F_{8, 40} = 3.081$, $p = 0.008$).

Biofilms on Coralline Species

The proportion of larvae choosing a CCA specimen in Choice Experiment 3 showed a weak positive correlation ($r = 0.52$, $P = 0.02$, $n = 25$) with the density of diatoms on that plant (Fig. 2A). In contrast, the proportion of settling larvae showed a nonsignificant negative correlation ($r = -0.25$, $P = 0.52$, $n = 25$) with the density of bacteria (Fig. 2B). Bacterial and diatom densities varied greatly between plants within species and between quadrats within plants. Mean diatom densities were low, ranging from zero to only 3×10^4 cm⁻² (Fig. 2A, Table 3). Bacterial densities ranged from 0.2 to 8×10^6 cm⁻² (Fig. 2B).

On *Mesophyllum prinzianum* there were clearly demarcated areas with either few bacteria and little surface polysaccharide, or many bacteria among copious polysaccharide (Fig. 4A to D). Diatoms were seen only as one patch on one of the five *Mesophyllum prinzianum* plants examined. *Mesophyllum prinzianum* previously collected from the same location had dense patches of diatoms in grazing refugia beside protruberances or in sunken conceptacle pore-plates (Fig. 4 E, F). However, searching with SEM confirmed the absence of such patches of diatoms on the plants used in this Choice Experiment 3.

Lithophyllum carpophylli from Experiment 3 also showed biofilm patchiness on a scale of hundreds of micrometres. Bacteria were much more abundant in the cavities formed by cells that had lost their exterior wall (Fig. 5A). Few bacteria were seen on areas of thallus where the external wall of the cells was flush with the thallus surface (no cell cavities present). However, on another occasion this did not hold true (Fig. 5B). No diatoms were seen on the plants of *Lithophyllum carpophylli* used in Choice Experiment 3, either in the 60 diatom quadrats (Fig. 2A), or in visual scanning the plants with SEM. On another occasion, variable densities of diatoms were found on this CCA species (Table 3).

Bacteria were several times less abundant on *Phymatolithon repandum* than on the other coralline species (Figs. 2B, 5C). The surface of this species typically had extensive fine debris that resulted from disintegration of the calcified lateral cell walls (Fig. 5C). Although this may obscure some bacteria from view, it was obvious that the bacterial density was low. Diatoms were found on only two of the five plants inspected, and the average diatom density was low (Fig. 2A). On other occasions, high diatom densities were seen on *Phymatolithon repandum*, but these appeared to be removed by epithallial sloughing (Fig. 5D). Extensive grazing marks were often visible on this species (Fig. 5E) and microbial densities were low in areas scarred by grazing.

Pneophyllum coronatum and *Hydrolithon rupestris* had variable densities of diatoms (Figs. 2A, B, Table 3) and bacteria (Fig. 5F, H). On *Pneophyllum coronatum* there were fewer microbes on young tissue close to the growing edge of the plant (Fig. 5G).

The diatom assemblage on most plants was dominated by *Cocconeis* spp., *Amphora* spp. and other naviculoid diatoms (Table 3). On one occasion *Tabularia* sp. was abundant in grazing refugia on *Mesophyllum prinzianum*.

No-choice Experiments With Coralline Species Assayed Individually

When the five species of CCA were presented individually to larvae, all induced a high proportion of larvae to attach and metamorphose. There were some differences in the timing of responses among CCA species and among experiments (Figs. 6, 7). Those species that induced metamorphosis most rapidly (*Pneophyllum coronatum* and *Hydrolithon rupestris*) (Figs. 6, 7) were the species preferred in the choice experiments (Figs. 1 and 3).

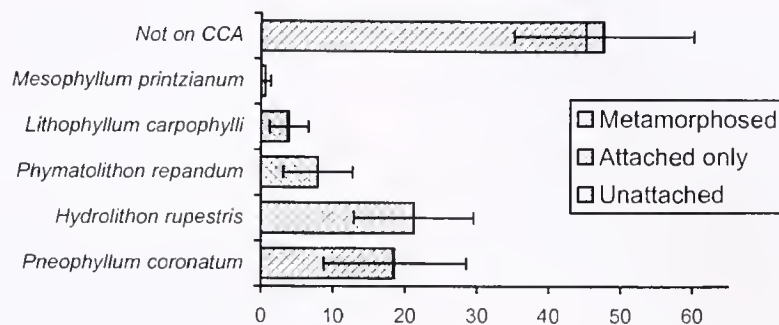
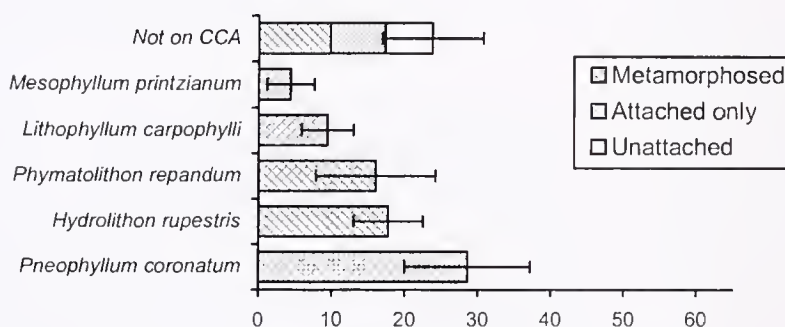
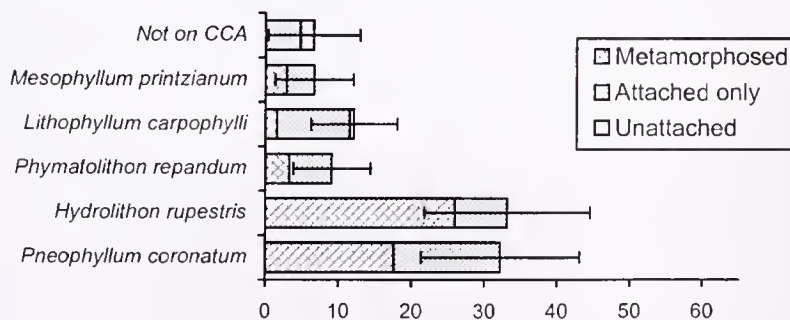
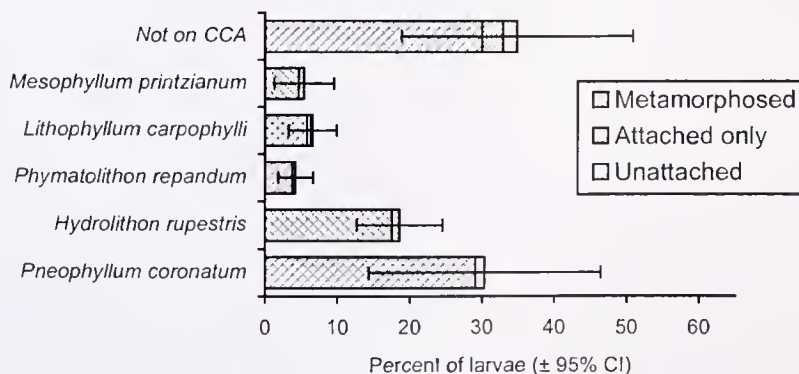
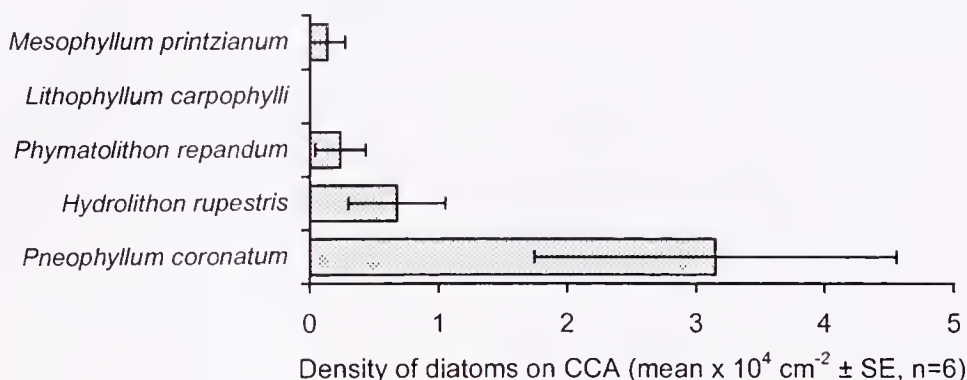
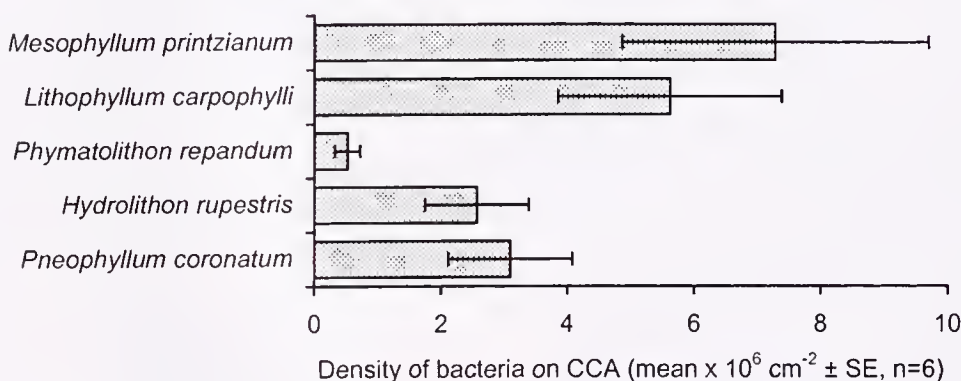
A) Choice Experiment 1: 22 hour counts**B) Choice Experiment 2: 19 hour counts****C) Choice Experiment 3: 23 hour counts****D) Choice Experiment 3: 69 hour counts**

Figure 1. Settlement of larvae offered a choice of five CCA species. Locations are categorized as being on one of the coralline algal species, or "not on CCA". Settlement stages are defined as Unattached = larvae have not attached or metamorphosed; Attached only = larvae have attached but not metamorphosed; Metamorphosed = larvae have initiated metamorphosis by shedding the velum and may or may not have commenced postlarval shell growth. Confidence intervals apply to the totals for each category. Data are shown for three experiments. For experiment 3, counts were made after both 23 and 69 h of bioassay.

A) Diatom density



B) Bacterial density



C) CCA fragment size

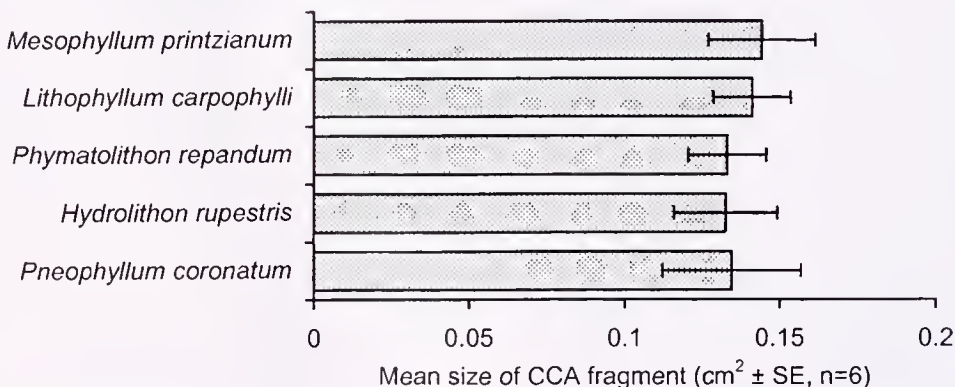


Figure 2. Covariates measured for Choice Experiment 3. Fragment size is measured as the "floor area" covered by each CCA fragment. Diatom and biofilm densities were determined by SEM inspection of quadrats on fragments of the same plants used in settlement bioassays (see Fig. 1C, D).

In the first experiment, the mean percent metamorphosis within 20 h was $\geq 95\%$ on *Pneophyllum coronatum* and *Hydrolithon rupestris*, but only 57% on *Mesophyllum printzianum* and 68% on *Phymatolithon repandum* (Fig. 6A). By 60 h, most of the remaining larvae had metamorphosed (Fig. 6B). The same pattern was evident in No-choice Experiment 3, where metamorphosis occurred more slowly (Fig. 7A, B).

Movement off CCA

The animals that were not on CCA after 20–24 h were mostly unmetamorphosed (Fig. 8). The exception was in Choice Experi-

ment 1, where a large proportion of larvae were found metamorphosed, and not on CCA (Fig. 1A). By 2.5 to 3 days there was an increase in the total number of larvae not on CCA and a predominance of animals with postlarval shell growth, which had metamorphosed on CCA then migrated off (Fig. 8).

DISCUSSION

Strong Settlement on all CCA Species Tested

When presented individually, all five species of coralline induced over 80% of *Haliotis iris* larvae to metamorphose within 3

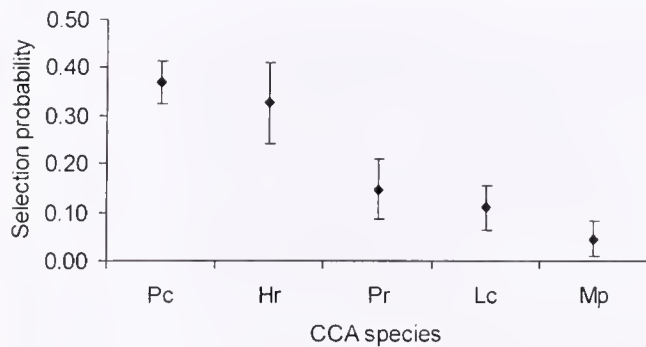


Figure 3. Probability of *Haliotis iris* larvae settling on each of the five CCA species when given a choice. Analysis used data from 1 day counts in Choice Experiments 1, 2, and 3. Pc = *Pneophyllum coronatum*, Hr = *Hydrolithon rupestris*, Pr = *Phymatolithon repandum*, Lc = *Lithophyllum carpophylli*, Mp = *Mesophyllum printzianum*.

days. There was variation in the speed of the response among CCA species, and between the two experiments (Figs. 5, 6) but all CCA species were effective inducers of metamorphosis. When presented with a choice of 5 CCA species, *Haliotis iris* larvae preferentially settled on certain species (Fig. 2), and the preferred species were those that induced metamorphosis most quickly in the no-choice experiments.

The strong settlement response of *Haliotis iris* larvae to all CCA tested contrasts with the major differences among CCA species reported for *Haliotis laevigata* (Daume et al. 1999) and a scleractinian coral (Morse et al. 1988), but is consistent with the response of *Haliotis rufescens* (Morse et al. 1980, Morse & Morse 1984) and a soft coral (Sebens 1983) to multiple CCA species.

Rejection Rates of CCA Underlie Settlement Preferences

Given that the chance of larval encounter is equivalent for the various CCA species (Fig. 2C), the existence of settlement preferences implies that larvae were more likely to reject certain species after contact. This preference was exercised even though all species were strong inducers of metamorphosis when presented alone (Figs. 6, 7). The more rapid metamorphosis seen on *Pneophyllum coronatum* and *Hydrolithon rupestris* in the no-choice experiments is consistent with the greater likelihood of larvae "accepting" these substrata when contacted by larvae.

Factors Controlling Acceptance or Rejection of CCA

There are various possible causes for the preferential settlement on certain CCA species. Some potential causes relate to factors that covary with CCA species, rather than being a function of the species itself.

The settlement preferences we observed could result from differences in the settlement-inducing chemicals of the different CCA species (McShane 1996). The specific chemicals involved, the quantities produced, or their availability at the surface of the plant could all vary within and among CCA species, but there is no direct evidence to support or refute this. The recent identification of an abalone metamorphosis-inducing molecule from a CCA (Suenaga et al. 2004) provides an analytical target to begin exploring this question.

The biofilm on CCA is a potential determinant of settlement inducing activity (Johnson et al. 1991, Johnson & Sutton 1994). In the present study, the proportion of larvae choosing a CCA speci-

men showed no correlation with bacterial density, but a weak positive correlation with diatom density. The density of diatoms on CCA was generally low, with even the highest densities barely reaching levels known to induce high levels of abalone larval attachment (Kawamura & Kikuchi 1992, Roberts 2001a). However, diatoms can combine synergistically with other cues (Roberts & Nicholson 1997, Roberts 2001a) and the densities required to be effective in such a synergism might be much lower.

There was a relationship between the morphology of the corallines and the settlement preferences in choice experiments. The two most preferred species (*Pneophyllum coronatum* and *Hydrolithon rupestris*) were both thin encrusting corallines, the intermediate choice (*Phymatolithon repandum*) was a slightly thicker encrusting to warty growth form, whereas the two least preferred species (*Lithophyllum carpophylli* and *Mesophyllum printzianum*) were thick crusts (Table 2). It is interesting to consider whether these choices might make any ecological sense. Thick crusts of *Mesophyllum printzianum* occur on stable rock substrata (Table 2, Naylor & McShane 1997a) whereas the thin encrusting *Pneophyllum coronatum* and *Hydrolithon rupestris* seem to be opportunistic species (author's personal obs.), such as found on mobile rocks in shallow water (McShane 1996, Naylor & McShane 1997a). Experimental evidence shows that the thick-crust *Mesophyllum printzianum* hosts polychaete predators that kill many recently settled abalone (Naylor & McShane 1997a, Naylor & McShane 1997b) and that its surface topography offers no demonstrable protection from dislodgement of *Haliotis iris* postlarvae by wave shear (Naylor & McShane 1997b). Juvenile *Haliotis iris* (10–70 mm shell length) are found during daylight hours under rocks that have undersides free of bulky encrusting organisms (McShane et al. 1995, author's personal obs.). Such rocks are somewhat mobile, occur in shallow water and are commonly colonized by thin crusts of CCA (McShane 1996, Naylor & McShane 1997a) similar to *Pneophyllum coronatum* and *Hydrolithon rupestris*. McShane (1996) remarked on the low frequency of *Haliotis iris* recruits on thick growth forms of CCA and suggested that lower concentrations of settlement inducers or the presence of predatory polychaetes were possible causes. Thus the preference for thin crusts that we observed in *Haliotis iris* larvae has potential benefits for both postlarvae and cryptic juveniles, though the suggestion of any link is merely speculative.

There has been some discussion about the effect that CCA growth form has on the biofilm present. Morphologically complex growth forms might protect areas from access by larger grazers, leaving remnants of biofilm as food for abalone recruits (Shepherd & Daume 1996, Day & Branch 2000) or as cues for larval settlement. This supposition is not supported by the observation that the lumpy coralline *Sporolithon durum* had no more biofilm than encrusting or warty forms (Shepherd & Daume 1996, Daume et al. 1999). However, the "lumps" on *Sporolithon durum* are several mm diameter, so would not restrict access for smaller grazers, and the sparse biofilm on this coralline probably results from its extensive epithallial sloughing (Shepherd & Daume 1996). In the present study, the effect of grazing refuges was clearly visible on some specimens of the fruticose *Mesophyllum printzianum*, where pockets of diatoms proliferated in the narrow gaps between protruberances or the sunken pore plates of conceptacles (Fig. 4E, F). Sloughing was also evident in some species (Fig. 5D) and contributed to the extreme variability in biofilm composition. This variability suggests that differences in biofilm characteristics

TABLE 3.

Composition of diatom assemblages on the CCA specimens used in Choice Experiment 3. Data are from 12 random quadrats on each of 5 plants viewed by SEM (see Methods).

CCA Species	Percentage of Total Diatom Cells Seen on Plant:					Number of Plants With Diatoms	Total Number of Diatoms	Density (Cells cm ⁻²)
	<i>Cocconeis</i> spp.	<i>Amphora</i> spp.	<i>Tabularia</i> spp.	<i>Achnanthes</i> spp.	Other Naviculoids			Mean ± SE
Experiment 3 plants								
<i>Pneophyllum coronatum</i>	81 ± 9	13 ± 9			6 ± 4	5	173	3.1 ± 1.4 × 10 ⁴
<i>Hydrolithon rupestris</i>	70 ± 24	30 ± 24			0 ± 0	4	40	6.8 ± 3.8 × 10 ³
<i>Phymatolithon repandum</i>	50 ± 50	25 ± 25			25 ± 25	2	14	2.4 ± 2.0 × 10 ³
<i>Lithophyllum carpophylli</i>	—	—			—	0	0	0.0 ± 0.0
<i>Mesophyllum printzianum</i>	0	0			100	1	8	1.4 ± 1.4 × 10 ³
Additional plants from same location on other dates								
<i>Pneophyllum coronatum</i>	51 ± 11	15 ± 8	1 ± 1	0 ± 0	33 ± 12	5	61	7.0 ± 3.4 × 10 ³
<i>Hydrolithon rupestris</i>	25 ± 22	54 ± 19	0 ± 0	0 ± 0	21 ± 11	4	9	1.2 ± 0.5 × 10 ³
<i>Phymatolithon repandum</i>	38 ± 10	2 ± 2	0 ± 0	1 ± 1	59 ± 10	4	97	1.6 ± 0.4 × 10 ⁴
<i>Lithophyllum carpophylli</i>	26 ± 11	0 ± 0	17 ± 13	0 ± 0	58 ± 23	3	201	3.4 ± 3.3 × 10 ⁴
<i>Mesophyllum printzianum</i>	37 ± 16	1 ± 1	57 ± 14	3 ± 3	3 ± 2	4	102	1.7 ± 0.3 × 10 ⁴

Data are mean percentage \pm SE, n = number of plants with diatoms.

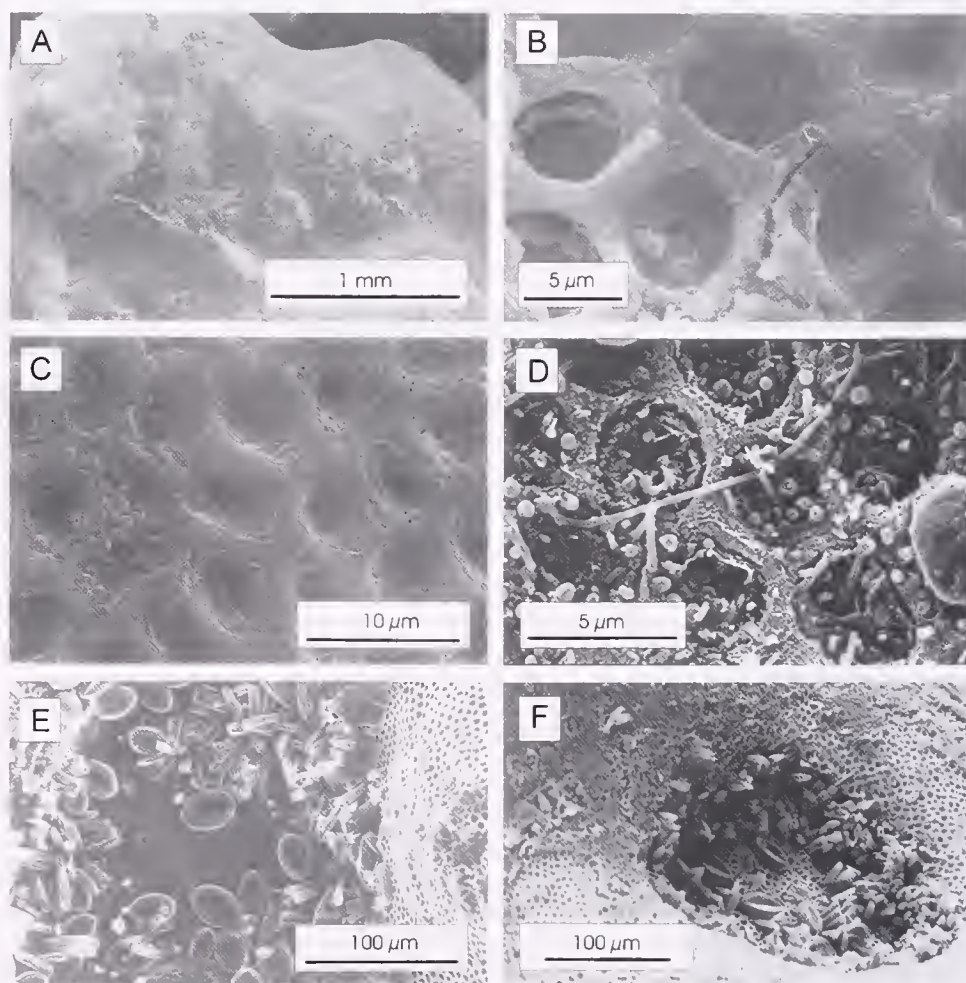


Figure 4. Selected features of the biofilm on *Mesophyllum printzianum*. A. Light and dark areas on *Mesophyllum printzianum* that are shown in photo B, to be patches with little surface polysaccharide and few bacteria (light areas) or copious polysaccharide and numerous bacteria. C. Bacteria and polysaccharide. D. SEM Method 2 of bacteria. E, F. Diatoms (*Tabularia* sp. and *Cocconeis pseudomarginata*) in grazing refugia.

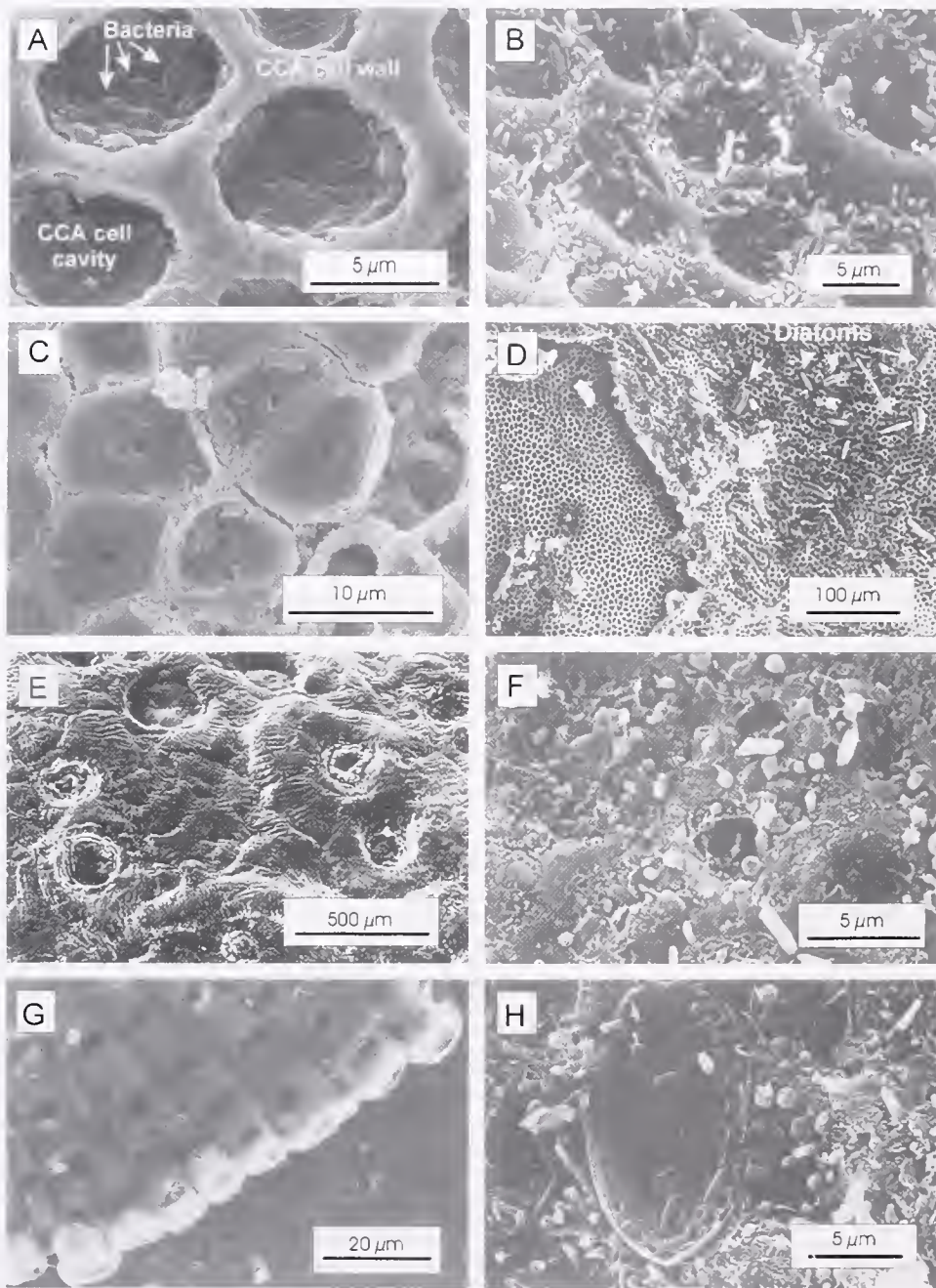


Figure 5. SEM photos of coralline surfaces. A. *Lithophyllum carpophylli* showing high densities of coccoid bacteria in the cavities of CCA cells that have lost their exterior wall, although, B. shows that this was not always the case. C. *Phymatolithon repandum* showing a few bacteria among the fine debris that results from the disintegration of calcified cell walls. D. *Phymatolithon repandum* – the tissue at the left of the photo was newly exposed by epithallial sloughing and clear of diatoms, whereas older tissue had many diatom cells. E. Extensive grazing marks on *Phymatolithon repandum*. F. *Hydrolithon rupestris* with a moderately high density of bacteria. G. The growing edge of *Pneophyllum coronatum*. H. Abundant bacteria and a basal valve of *Cocconeis* sp. on *Pneophyllum coronatum*.

among CCA species are unlikely to explain consistent patterns in abalone settlement or postsettlement survival.

The CCA species used in the present study were from different locations, or different microhabitats within those locations (Table 2). Thus environmental effects such as growing conditions or grazing history could affect settlement preferences. Grazing could affect the inducing activity of CCA species by causing cell damage, allowing access to intracellular inducer molecules. CCA tissue can

be broken by the grazing of abalone (Shepherd & Cannon 1988, Shepherd & Daume 1996) and various other invertebrate grazers (Steneck & Watling 1982). In order for grazing damage to explain settlement preferences for certain CCA, the grazing would need to differentially affect the CCA species. In the present study grazing damage was evident on *Phymatolithon repandum* (Fig. 5E) and *Pneophyllum coronatum* but not on the favored *Hydrolithon rupestris*. CCA cell damage from grazing need not result in higher larval

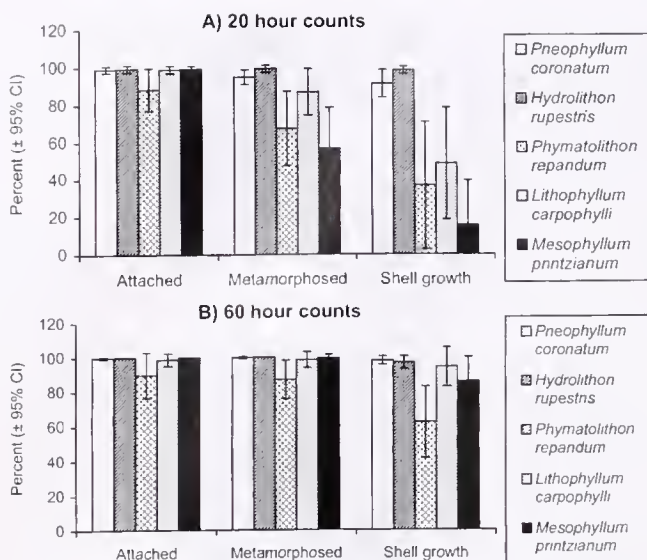


Figure 6. Settlement response of larvae presented with a single species of coralline alga (No-choice Experiment 1). Settlement stages are Attached = larvae attached by their foot including those that have metamorphosed; Metamorphosed = larvae have shed their velum and may or may not have commenced postlarval shell growth; Shell growth = larvae that have shed the velum and initiated postlarval shell growth.

settlement. Daume et al. (1999) found that *Haliotis laevis* larvae settling on the CCA *Sporolithon durum* lined up along the edges of cuts in the thallus, but the number of larvae that chose damaged plants was not significantly higher than that choosing undamaged plants. Johnson et al. (1991) found no difference in the number of *Acanthaster planci* larvae settling on damaged and undamaged *Lithothamnium pseudosorum*.

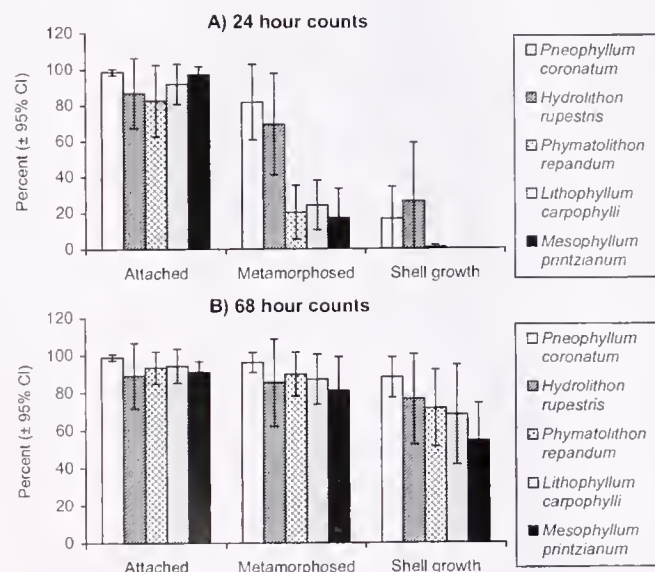


Figure 7. No-Choice Experiment 3: Settlement response of larvae presented with a single species of coralline alga (no choice). Settlement stages are defined as Attached = larvae attached by their foot including those that have metamorphosed; Metamorphosed = larvae have shed their velum and may or may not have commenced postlarval shell growth; Shell growth = larvae that have shed the velum and initiated postlarval shell growth.

Minimal Effect From Dissolved Inducers in Assays

In the present study, the corallines were broken into fragments for the experiments, so the specimens were not intact. Thus active chemicals may have leaked from the plants into the assay water. Water-soluble extracts of *Phymatolithon repandum* induce larval attachment, and sometimes metamorphosis, in *Haliotis iris* (Roberts & Nicholson 1997, R. Roberts unpubl. data). The concentration required to induce 50% larval attachment is generally between 100 and 200 μg dry extract per mL of water (R. Roberts unpubl. data). It is unlikely that this concentration would be produced by leakage from damaged edges in the settlement assays, but some effect from soluble inducers cannot be ruled out. Water soluble inducers would cause larvae to cease swimming and begin crawling within several minutes (R. Roberts unpubl. data). Crawling larvae may find access easiest for those corallines supported on flat plastic sheet (*Pneophyllum coronatum* and *Hydrolithon rupestris*), and this differential accessibility could confound results. In Choice Experiment 2, the CCA fragments were soaked in FSW for 4 days before assayed to reduce any effect from leaking cell contents. Settlement preferences were not appreciably altered (Fig. 1A, B). Experiment 3 tested the accessibility issue by pressing the coralline samples into clay so that the live surface of each specimen was flush with the surrounding substratum and all plants were equally accessible to crawling (or sinking) larvae. Larvae still displayed the same preference for *Pneophyllum coronatum* and *Hydrolithon rupestris* over the remaining species.

Timing of Observations in Settlement Choice Experiments

To detect preferential settlement, counts had to be made early enough to precede the movement of postlarvae off CCA, but late enough to let larvae respond to the CCA. Counts made after 1 day were informative despite the variation in speed of metamorphosis between larval batches. In all cases there was some movement of metamorphosed animals off CCA between 1 and 3 days after settlement (Fig. 8). *Haliotis iris* remain on the upper surfaces of CCA-coated boulders for a few months after settlement (McShane & Naylor 1995) so their movement off CCA in the present study would not be in search of shelter or darkness. It is likely to be caused by food depletion, as the experiments used high densities of larvae (Table 1) on small fragments of CCA (e.g., Fig. 2C). Newly metamorphosed postlarvae are capable of eating small, loosely attached particles including diatoms and bacteria (Seki & Kan-no 1981, Norman-Boudreau et al. 1986, Roberts et al. 1999a, b), and 6-wk old postlarvae can ingest the cuticle and epithelial cells of CCA (Garland et al. 1985). Copious fecal pellets (devoid of diatoms and CCA cells) are visible around CCA-coated pebbles within 2 days of settlement induction (R. Roberts, pers. obs.). This extracellular food source would probably be quickly depleted at high postlarval densities.

How Should Target Size be Measured?

Competent abalone larvae periodically cease swimming, sink to the bottom, pull themselves onto their foot. They may crawl some distance while testing the surface for the presence of settlement cues (Seki & Kan-no 1981). The larvae require surface contact to respond to intact corallines, rather than showing taxis toward the source of a water-borne cue (Morse et al. 1980). The appropriate "target size" for larvae sinking through the water column is the "floor area" covered by the CCA fragment, as measured here. For crawling larvae, the distance around the perimeter of the fragment

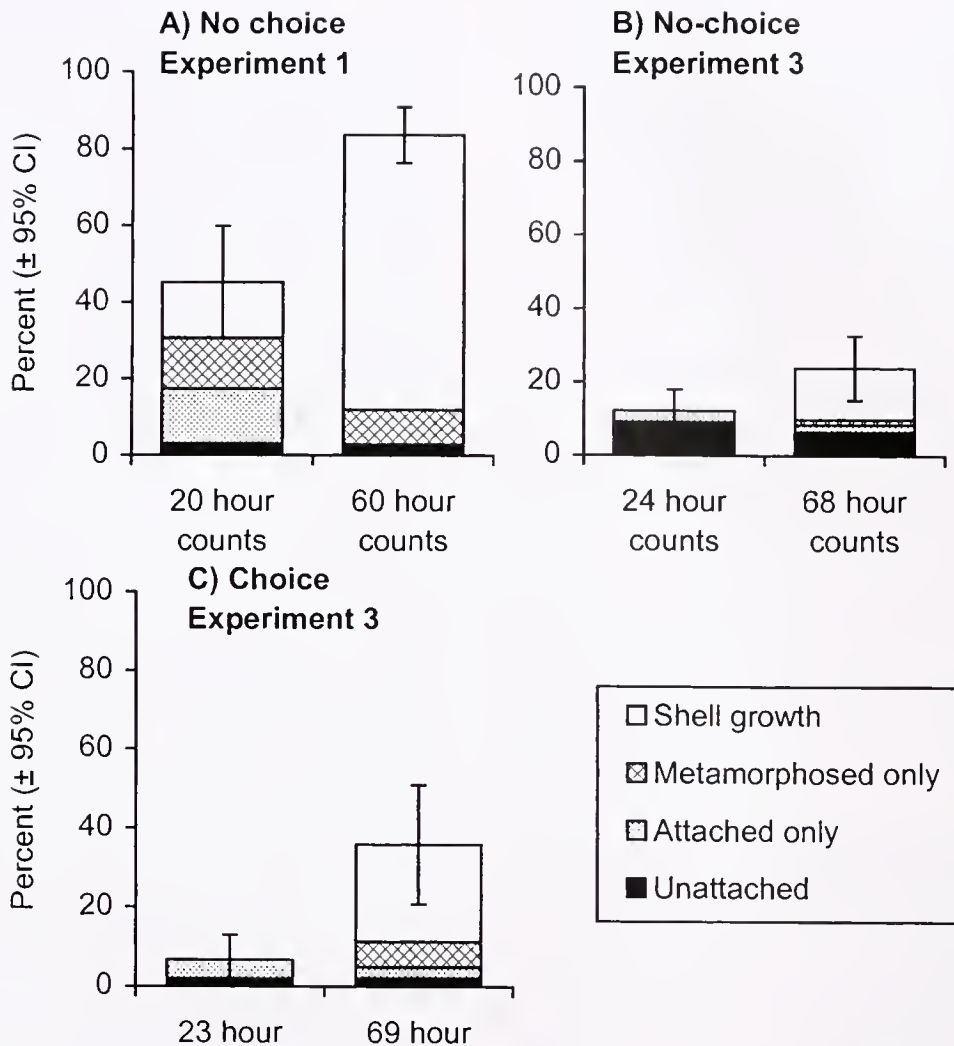


Figure 8. Number and developmental stage of abalone found "not on CCA" after ~1 day and ~3 days of bioassay. Settlement stages are defined as Unattached = larvae have not attached or metamorphosed; Attached only = larvae have attached but not metamorphosed; Metamorphosed only = larvae that have shed the velum but not grown any postlarval shell; Shell growth = larvae that have shed the velum and initiated postlarval shell growth. Confidence intervals apply to the totals for each column.

is most relevant, but the floor area measurement is a reasonable approximation for objects that are not long and thin.

CONCLUSION

Although the five species of CCA tested were all effective inducers of *Haliotis iris* larval attachment and metamorphosis, larvae did exhibit a preference for certain species when given a choice. This selection of certain substrata most likely results from different probabilities of acceptance or rejection following contact with a substratum. This study emphasizes the strong effect of selective settlement even among a suite of highly effective settlement substrata. From these laboratory results it is hypothesized that recruitment of *Haliotis iris* in the wild would vary among CCA species

or growth forms, and that this variation is at least partly determined at the time of larval settlement.

ACKNOWLEDGMENTS

The authors thank Sabine Daume and Bill Woelkerling for tuition and expertise on corallines identification, and for helpful discussions; Christine Handley for technical assistance; Mark Gould for guidance on scanning electron microscopy and Mike Barker and Philip Mladenov for review of drafts. This research was supported by contracts CAW 801 and CAWX0004 with the New Zealand Foundation for Research Science and Technology and by a University of Otago Alliance Group Scholarship.

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EFFECT OF GRAZING BY A HERBIVOROUS GASTROPOD *HOMALOPOMA AMUSSITATUM*, A COMPETITOR FOR FOOD WITH POST-LARVAL ABALONE, ON A COMMUNITY OF BENTHIC DIATOMS

TOMOHIKO KAWAMURA,^{1,*} HIDEKI TAKAMI,² AND YOH YAMASHITA³

¹Ocean Research Institute, The University of Tokyo, Nakano, Tokyo 164-8639, Japan; ²Tohoku National Fisheries Research Institute, Shiogama, Miyagi 985-0001, Japan; ³Maizuru Fisheries Research Station, Field Science and Education Research Center, Kyoto University, Maizuru, Kyoto 625-0086, Japan

ABSTRACT The effect of grazing by a small herbivorous gastropod *Homalopoma amussitatum* on the density and species composition of benthic diatoms was examined in the laboratory. *H. amussitatum* is a dominant species on exposed crustose coralline algae (CCA) along the Sanriku coast in northeastern Japan, and it has been suggested that it competes for food with postlarval abalone. The minimum densities of the gastropod required to inhibit the growth of diatoms were estimated to be <10 g/m² at 20°C, between 10–20 g/m² at 15°C, and >20 g/m² at 7°C. In the absence of grazing pressure, *Nitzschia* sp. and *Psamodictyon panduriformis*, which have a growth form classified as Type A (gliding prostrate type), initially formed flat communities. *Amphora* sp., *Cocconeis sublittoralis* (Type B: adhesive prostrate type) and *Tabularia investiens* (Type C: nonmotile upright type) subsequently increased in number. The two species of Type A were significantly affected by grazing pressure and did not increase even at a gastropod density of 10 g/m² at 15°C and 20°C. Types B and C diatoms were more tolerant of grazing by the gastropod than Type A species. However, the volumetric rate of ingestion of *Cocconeis scutellum* (Type B) by the gastropod was almost the same as for *Navicula britannica* (Type A) at the three experimental temperatures, when they were supplied to the gastropod independently. The digestion efficiencies of *C. scutellum* and *N. britannica* were 94.7% and 2.2%, respectively. The dietary value of *C. scutellum* for the gastropod *H. amussitatum* is thus considerably higher than that of *N. britannica*. In the natural CCA habitat, competitive grazing by *H. amussitatum* on the diatom *Cocconeis* spp., which is known as a major food for postlarval abalone, could significantly affect the survival and growth rates of postlarval abalone.

KEY WORDS: benthic diatom, grazing, gastropod, post-larval abalone, competition

INTRODUCTION

Benthic diatoms are the principle food for postlarval abalone (e.g., Kawamura 1996, Kawamura et al. 1998c). The abundance and species composition of benthic diatom communities on crustose coralline algae (CCA), where abalone larvae predominantly recruit (e.g., Saito 1981, Morse & Morse 1984), are suggested to significantly affect the survival and growth of metamorphosed abalone (Kawamura et al. 1998c, Takami et al. 1997, Takami et al. 2000). However, there is little information on the diatom communities on CCA surfaces in natural habitats. On CCA, there are many herbivores other than abalone (e.g., Asano et al. 1990, Kawamura et al. 1992), and their grazing controls the benthic diatom communities colonizing CCA (Kawamura 1994a, Kawamura 1994b). Herbivorous gastropods were determined to be the main grazers on benthic diatoms, controlling their abundance and species composition on CCA at several sites along the Sanriku coast in northeastern Japan (Kawamura et al. 1992, Kawamura 1994b, Takami 2002). When the densities of gastropods were high, diatom densities were low and grazer-tolerant species dominated regardless of other environmental factors (Kawamura et al. 1992, Kawamura 1994b). Three-dimensional communities developed only when the densities of gastropods were low (Kawamura et al. 1992, Kawamura 1994b). The effect of grazing by herbivorous gastropods on microalgal composition has been studied qualitatively (e.g., Nicotri 1977, Suzuki et al. 1987, Steinman et al. 1989) but not quantitatively.

A small herbivorous gastropod *Homalopoma amussitatum* Gould 1861 is a dominant species on CCA along the Sanriku coast in northeastern Japan (Kawamura et al. 1992, Kawamura 1994b,

Takami 2002) and has been suggested that it competes for food with postlarvae of the abalone *Haliotis discus hannai* Ino 1953 (Takami et al. 2001). Postlarval *H. discus hannai* reared with *H. amussitatum* showed significantly slower growth than postlarvae reared with 1-y-old *H. discus hannai* (Takami et al. 2001). There was no significant difference in the overall density of benthic diatoms between aquaria with *H. amussitatum* and with 1-y-old *H. discus hannai*, but the composition of diatom species differed among aquaria. The diatoms with a high dietary value for postlarval abalone, such as *Cocconeis* spp. (Kawamura et al. 1998c), had higher densities in aquaria with 1-y-old abalone than in aquaria with *H. amussitatum* (Takami et al. 2001).

In this study, the effect of grazing by *H. amussitatum* on the density, species composition and community structure of benthic diatoms was examined quantitatively in the laboratory. Benthic diatom communities are composed of species with various growth forms (Kawamura & Hirano 1992). Growth form influences tolerance of grazing (Kawamura & Hirano 1992, Kawamura 1994a, Kawamura 1994b) and affects ingestibility and digestion efficiency of diatoms for grazers, thus determining their nutritional value (Kawamura et al. 1995, 1998b, Roberts et al. 1999). Diatom species have been divided into 8 types (Types A to H) of growth form (see Materials and Methods). Feeding rates of the gastropod on 2 monoculture diatom strains with different growth forms (*Navicula britannica* Husted et Aleem 1951: Type A, *Cocconeis scutellum* Ehrenberg 1838: Type B) were measured. Diatoms with a Type A growth form are generally readily grazed, whereas those with Type B are less affected by grazing (Kawamura & Hirano 1992, Kawamura 1994a, Kawamura 1994b). However, Type A diatoms, which have low attachment strength, were easily ingested by postlarval abalone *H. discus hannai* (Kawamura et al. 1995) and *H. iris* Gmelin 1791 (Kawamura et al. 1998b) without cell rupture. The majority of ingested cells passed through the gut alive

*Corresponding author. E-mail: kawamura@ori.u-tokyo.ac.jp

and unharmed, resulting in low digestion efficiencies and thus low abalone growth rates (Kawamura et al. 1995, Kawamura et al. 1998b). The digestibility of diatom cells has only been examined for abalone. The digestion efficiency of two diatom strains for *H. amussitatum* was examined. We discuss the results of the present study in light of the competition for food between post-larval abalone and *H. amussitatum* previously reported by Takami et al. (2001).

MATERIALS AND METHODS

Effect of Grazing on Benthic Diatom Communities

Diatom communities colonizing glass slides were held in aquaria ($17 \times 19 \times 17.5 \text{ cm}^3$) under natural light conditions at Tohoku National Fisheries Research Institute, in Shiogama, Miyagi, Japan. Aquaria were supplied with sand-filtered and temperature-controlled seawater (1.1–1.2 L/min.) at 7°C, 15°C, and 20°C. Glass slides were set on the bottom of each aquarium. Adult *H. amussitatum* were kept at 4 biomasses (0, 10, 20, 40 g/m²). The gastropods were collected in the coastal waters off Eno-shima Island and were acclimated to the experimental seawater temperatures in advance. The shell height and wet weight of gastropods ranged between 5.0–11.0 mm ($7.5 \pm 0.1 \text{ mm}$; mean \pm standard error) and 0.1–0.6 g ($0.22 \pm 0.03 \text{ g}$), respectively. Two glass slides from duplicate aquaria were collected at 7–10-day intervals over 45 days. Density, species, composition, and growth form of diatoms colonizing the upper surface of the glass slides were examined under a light microscope. Diatom species were identified by a scanning electron microscope after acid cleaning (Takano & Nagumo 1987).

Growth forms of benthic diatoms are classified into 8 types (Types A to H) based on their mode of attachment, whether solitary or colonial forms, and by their motility and adhesive strengths (Kawamura & Hirano 1992, modified by Kawamura 1994a). The major diatom species colonizing glass slides in the present experiment were divided into the 4 types of growth form (Types A, B, C, and E). Type A (gliding prostrate type): solitary cells with a prostrate form, swift gliding movements, and very low adhesive strength. Type B (adhesive prostrate type): solitary cells with a prostrate form, slow movement, and high adhesive strength. Type C (nonmotile upright type): nonmotile solitary cells or simple, fan-shaped colonies standing upright with a relatively weak adhesion to the substratum. Type E (mucous thread solitary type): solitary cells or short, belt-shaped colonies attached strongly to the substratum with a mucous thread.

The differences in diatom density among experimental treatments were tested using Tukey-Kramer multiple comparison test.

Feeding Rates on Two Diatom Species

Feeding rates of *H. amussitatum* were measured individually at 7°C, 15°C, 20°C on 2 monoculture diatom strains (*Navicula britannica*: Type A, *Cocconeis scutellum*: Type B) by counting the diatom cell number in the feces. Cell volume of the diatoms, *N. britannica* and *C. scutellum* were $2343.4 \mu\text{m}^3$ ($55.4 \times 9.0 \times 4.7 \mu\text{m}$) and $730.8 \mu\text{m}^3$ ($22.0 \times 15.1 \times 2.2 \mu\text{m}$), respectively. Gastropods ($6.4 \pm 0.2 \text{ mm}$ in shell height, $0.16 \pm 0.01 \text{ g}$ in wet weight) were reared individually, in triplicate at each temperature, in deep petri dishes (9 cm in diameter, 2 cm in depth) with diatoms growing on the bottom. All fecal pellets released by the gastropods were collected at 12- or 24-h intervals. Diatom cell numbers in the feces

were calculated from the number of valves counted with a light microscope after acid cleaning (Takano & Nagumo 1987). Gut passage time of diatoms was measured by changing diatom species, and measuring the time before new diatoms appeared in the feces.

Recently released fecal material of *H. amussitatum* ($n = 3$ for each diatom strain) reared at 20°C was observed with an inverted microscope, and the proportions of intact versus ruptured diatom cells in feces were counted. A parallel count was made of live versus ruptured cells in the diatom culture from which the fecal material was grazed. Digestion efficiency (%) was calculated as: $(1 - L/L_0) \times 100$, where L_0 is the proportion of live cells in the source culture, and L is the proportion of live cells in the fecal pellet.

RESULTS

Effects of Grazing on Benthic Diatom Communities

At 20°C, *Nitzschia* sp. (Type A) initially formed flat communities in the absence of grazing by the gastropod and significantly increased at Day 15 (Fig. 1, $P < 0.05$). After Day 25 *Amphora* sp. (Type B) significantly increased ($P < 0.05$) and dominated. After Day 35 *Tabularia investiens* (Wm. Smith) Williams and Round 1986 (Type C) had significantly increased ($P < 0.05$) in number and was the second-most dominant species. *Achnanthes brevipes* var. *intermedia* (Kützinger) Cleve 1895 (Type E) also slightly increased in number by Day 35 but was not abundant. *Nitzschia* sp. and *Amphora* sp. almost disappeared in aquaria with gastropods ($10\text{--}40 \text{ g/m}^2$) at 20°C. *Cocconeis sublitoralis* Hendey 1951 (Type B) also decreased in number under grazing pressure. The density of *Cocconeis scutellum* was higher in aquaria with gastropods ($10\text{--}40 \text{ g/m}^2$) than without gastropods (0 g/m^2). At Day 45 *C. scutellum* was dominant in aquaria with gastropods at 20 and 40 g/m² ($P < 0.05$). Remarkably, the density of *T. investiens* increased after Day 35 in aquaria with gastropods at 10 g/m² ($P < 0.05$) to be comparable in aquaria without gastropods. This diatom de-

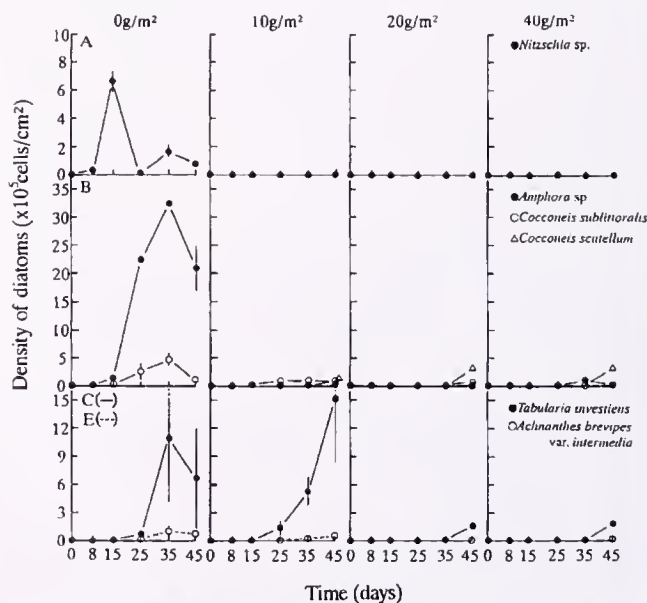


Figure 1. Changes in density of diatoms classified into growth forms (A, B, C, E) with four densities of *Homalopoma amussitatum* (0, 10, 20, 40 g/m²) at 20°C. Bars indicate standard errors of the mean ($n = 4$).

creased in number in aquaria with gastropods at 20 and 40 g/m² ($P < 0.05$), but was second-most dominant after *C. scutellum* at Day 45 ($P < 0.05$) (Fig. 1).

At 15°C, successions of diatom communities in aquaria without grazing (0 g/m²) were similar to those at 20°C, although growth rates of diatoms were lower (Fig. 2). *Nitzschia* sp. was most affected by grazing, and did not increase in aquaria with gastropods at 20 and 40 g/m² ($P < 0.05$). The density of *Amphora* sp. was significantly lower in aquaria with gastropods than without ($P < 0.05$), but relatively high in aquaria with gastropods at 10 g/m² after Day 35 ($P < 0.05$). *Psammодиетон panduriforme* (Gregory) Mann 1990 (Type A) and *C. sublittoralis* were generally less abundant in aquaria with gastropods, but their density in aquaria with 10 g/m² of gastropods was significantly higher than those in aquaria without gastropods after Day 35 ($P < 0.05$). *T. investiens* increased to a high density in aquaria with gastropods at 10 g/m² after Day 35 ($P < 0.05$), whereas it was not abundant in aquaria without gastropods (Fig. 2) ($P < 0.05$).

Densities of the 3 dominant species (*Nitzschia* sp., *Amphora* sp., and *C. sublittoralis*) at 7°C were all lower in aquaria with gastropods than those in aquaria without gastropods (Fig. 3). However, the densities of diatoms were less affected by grazing at 7°C than at higher temperatures, and the diatoms gradually increased in number in aquaria with gastropods at 10 and 20 g/m² ($P < 0.05$). In aquaria with gastropods at 40 g/m², only the density of *C. sublittoralis* increased slightly (Fig. 3), but there was no significant difference in the density of *C. sublittoralis* during the experimental period ($P > 0.05$).

Feeding Rates on Two Diatom Species

Gut passage time of diatoms was longer at lower temperatures but within 24 h even at 7°C. Because the gastropods were feeding and producing fecal material almost continuously, the number of

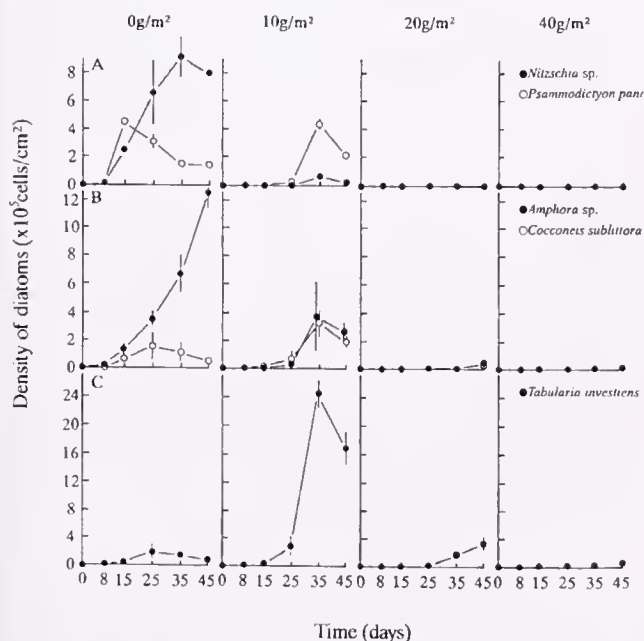


Figure 2. Changes in density of diatoms classified into growth forms (A, B, C) with 4 densities of *Homalopoma amussitatum* (0, 10, 20, 40 g/m²) at 15°C. Bars indicate standard errors of the mean ($n = 4$).

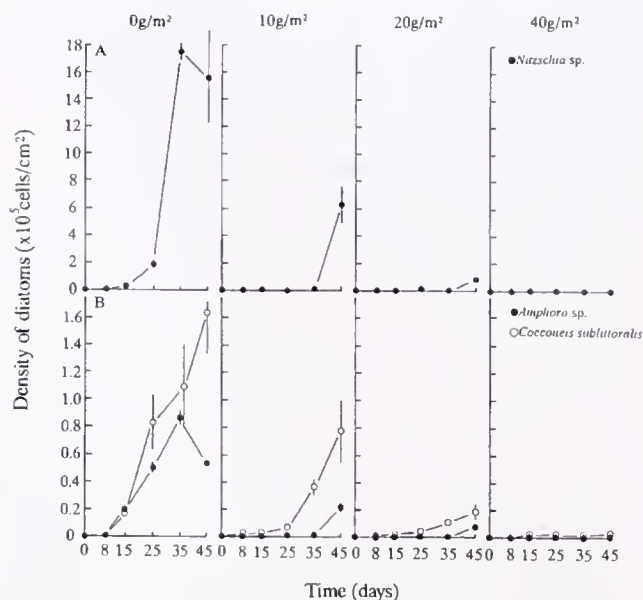


Figure 3. Changes in density of diatoms classified into growth forms (A, B) with four densities of *Homalopoma amussitatum* (0, 10, 20, 40 g/m²) at 7°C. Bars indicate standard errors of the mean ($n = 4$).

diatom cells in feces excreted in 24 h was considered to be equal to the amount of material ingested daily by an individual gastropod. The amount of fecal material gradually increased during the first 3 days and was almost constant after Day 3. The ingestion rate for the three seawater temperatures, calculated from the number of diatom cells excreted between Day 3 and Day 5, is shown in Table 1. The volume of each diatom strain ingested per g gastropod per day was almost the same, although the daily number of *N. britannica* cells ingested was less than *C. scutellum*. The digestion efficiency of *C. scutellum* at 20°C was 94.7%, whereas that of *N. britannica* was 2.2% (Table 1).

DISCUSSION

The succession of diatom communities, from Type A species (*Nitzschia* sp. and *P. panduriforme*) to Type B (*Amphora* sp. and *C. sublittoralis*) and Type C (*T. investiens*), dramatically changed with grazing by *H. amussitatum*. *Nitzschia* sp. and *P. panduriforme* of Type A growth form were affected more by grazing than diatoms of Types B and C (Figs. 1–3). It has been reported that

TABLE 1.

The ingestion rate and digestion efficiency of two diatom strains grazed by *Homalopoma amussitatum* at three temperatures.

Temperature (°C)	Diet	Ingestion rate		Digestion Efficiency (%)
		(Cells/g · Day, Mean ± SE)	(μm ³ /g · Day, Mean)	
7	<i>C. scutellum</i>	(1.5 ± 0.3) × 10 ⁶	1.1 × 10 ⁹	
	<i>N. britannica</i>	(3.0 ± 1.2) × 10 ⁵	7.0 × 10 ⁸	
15	<i>C. scutellum</i>	(6.4 ± 0.9) × 10 ⁶	4.7 × 10 ⁹	
	<i>N. britannica</i>	(1.5 ± 0.2) × 10 ⁶	3.5 × 10 ⁹	
20	<i>C. scutellum</i>	(8.3 ± 1.9) × 10 ⁶	6.1 × 10 ⁹	94.7
	<i>N. britannica</i>	(2.6 ± 0.3) × 10 ⁶	6.1 × 10 ⁹	2.2

Type A species are susceptible to being ingested by gastropods such as abalone (Ioriya & Suzuki 1987, Suzuki et al. 1987) and amphipods (Kawamura & Hirano 1992). The results of the present experiment showed that the two species of Type A growth form were most easily grazed by *H. amussitatum*.

At 15°C, the density of *T. investiens* in aquaria with gastropods at 10 g/m² was significantly higher than those in aquaria without gastropods after Day 35 (Fig. 2). At 20°C, the density of *T. investiens* in aquaria with gastropods at 10 g/m² also increased to be comparable in aquaria without gastropods (Fig. 1). The growth form of this species is easily grazed, and appeared to be abundant when grazing pressure was low in Tomarihama Bay in Miyagi (Kawamura et al. 1992). On the contrary, the density of this species was high under relatively high grazing pressure in Yoshihama Bay in Iwate (Kawamura 1994b). The dominant gastropod species were *Omphalius rusticus* Gmelin 1791 in Tomarihama Bay and *H. amussitatum* in Yoshihama Bay. Probably because *H. amussitatum* does not prefer to graze *T. investiens* (for unknown reasons other than growth form), this diatom appeared to increase when other diatoms were reduced by grazing. However, densities of this diatom were low in aquaria with gastropods at 20 and 40 g/m² (Figs. 1 and 2). It is suggested that *H. amussitatum* grazes *T. investiens* when food sources are limited.

Because the relative abundance of diatom species with a Type B growth form was high when the density of gastropods was high in Tomarihama Bay (Kawamura et al. 1992) and Yoshihama Bay (Kawamura 1994b), this type of diatom seemed to be relatively tolerant to grazing. The results of the present experiment showed that densities of *Amphora* sp. and *C. sublittoralis* (Type B) markedly decreased under the influence of grazing by *H. amussitatum*. On the other hand, *C. scutellum* of Type B appeared in relatively high density when grazed at 20°C (Fig. 1). A similar phenomenon was observed on settlement plates of abalone larvae in hatcheries (Ioriya & Suzuki 1987, Suzuki et al. 1987). This species may be able to increase growth under relatively high grazing pressure and/or when other diatoms are reduced by grazing, like *T. investiens*. However, the daily volume of *C. scutellum* cells ingested by the gastropod was almost the same as that of *N. britannica* cells when each diatom strain was supplied independently (Table 1). The digestion efficiency of *C. scutellum* was high, whereas most of *N. britannica* cells ingested by the gastropods passed through the gut alive and unbroken (Table 1). Although growth and nutritional condition of the gastropods were not examined in this study, *C. scutellum* seems to be a much more efficient diet than *N. britannica* for the growth of *H. amussitatum*, as previously found for abalone (Kawamura et al. 1995, Takami et al. 1996, Roberts et al. 1999). Many gastropods, such as abalone and *H. amussitatum*, probably graze all the microalgae growing on substrata regardless of their dietary value, with exceptions like *T. investiens*. Diatoms with Type B growth form are relatively hard to graze because they require considerable force to be removed, but if dislodged their cells are usually broken and release cell contents (Kawamura et al. 1995, Kawamura et al. 1998b, Roberts et al. 1999). For gastropods that can detach Type B diatom cells from substrata, these diatoms are efficient food sources because the diatom cell contents can be utilized.

Post-larval abalone can initially grow on biofilm materials including extracellular substances of diatoms, but larger postlarvae (>0.6–0.8 mm SL) need to use diatom cell contents for adequate growth (Kawamura et al. 1998c, Roberts et al. 1999). By approxi-

mately 0.8 mm SL postlarval *H. discus hannai* can detach *Cocconeis* cells efficiently, probably due to morphologic changes in the radula (Kawamura et al. 2001). Grazer-resistant algae such as Type B diatoms tend to dominate on CCA surfaces due to the high grazing intensity by gastropods. Dense patches of *Cocconeis* spp. were observed on CCA where postlarval and juvenile abalone were present (Takami 2002). Postlarval abalone that settled on CCA seemed to feed and grow mainly on *Cocconeis*. However, juvenile abalone of larger than 5–10 mm SL do not graze *Cocconeis* species if more favorable foods are available (Kawamura et al. 1998c), probably because the energetic value of *Cocconeis* ingestion becomes insufficient to support rapid growth due to their low-volume cells, prostrate growth form (Takami et al. 1996), and single layered colonies (Kawamura et al. 1998a). *H. discus hannai* larger than 1.8 mm SL use juveniles of the macroalga *Laminaria japonica* Areschoug 1851 efficiently (Takami et al. 2003). Juvenile and adult abalone graze 3-dimensional colonies of diatoms and loosely attached diatom cells simultaneously with their favored juvenile macroalgae, leaving behind prostrate diatoms with high attachment strength. Therefore *Cocconeis* spp. are often dominant on abalone larval settlement plates, which are pregrazed by conspecific juvenile abalone in hatcheries (Ioriya & Suzuki 1987, Suzuki et al. 1987, Seki 1997). It is considered that juvenile and adult abalone do not compete for food with postlarval abalone. On the other hand, the results of this study show that adult *H. amussitatum* is potentially a strong competitor for *Cocconeis* with postlarval abalone on CCA, as also suggested by Takami et al. (2001).

From the results of this study, the minimum densities of the gastropod *H. amussitatum* required to inhibit the growth of diatoms (except *T. investiens*) were estimated to be below 10 g/m² at 20°C, between 10 and 20 g/m² at 15°C, and above 20 g/m² at 7°C. On rocks covered by CCA in Tomarihama Bay, the density of herbivorous gastropods dominated by *H. amussitatum* was between 9.0 and 28.6 g/m² in 1989, and over 20 g/m² during the spawning season of the abalone *H. discus hannai* between August and October (Kawamura et al. 1992). Because the seawater temperature was over 15°C, most benthic diatoms that colonized CCA surfaces were probably eaten by gastropods. Postlarval abalone that settled on the CCA surfaces were unlikely to get sufficient food. During the study period in Tomarihama Bay, we did not collect any juvenile wild abalone from CCA rocks, although adult abalone and released juveniles lived nearby.

Sasaki and Shepherd (2001) reported that significant numbers of postlarval *H. discus hannai* of 5–7 days old, collected from boulders covered by CCA in Samenoura Bay, Miyagi in 1996 and 1997, showed an atrophied condition due to starvation. They hypothesized that the abundance of postlarvae of an herbivorous gastropod *Tegla* in the same habitat might cause food limitation and thus low survival of postlarval abalone. The results of the present study and Takami et al. (2001) indicated that a high density of gastropods such as *H. amussitatum* may affect abalone recruitment through competition for food.

ACKNOWLEDGMENTS

The authors thank Masamitsu Asano for useful suggestions during the experiments, and Christopher B. Clarke and two anonymous reviewers for constructive comments of a draft manuscript.

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GROWTH AND SURVIVAL OF JUVENILE RED ABALONE (*HALIOTIS RUFESCENS*) FED WITH MACROALGAE ENRICHED WITH A BENTHIC DIATOM FILM

JORGE ARTURO SIMENTAL, MARÍA DEL PILAR SÁNCHEZ-SAAVEDRA,* AND NORBERTO FLORES-ACEVEDO

Laboratorio de Biología y Cultivo de Microalgas, Departamento de Acuicultura, Centro de Investigación Científica y de Educación Superior de Ensenada, Apdo. Postal 2732, C.P. 22860 Ensenada, Baja California, México.

ABSTRACT The objective of this work was to evaluate the growth rate of 150-day-old juvenile abalone *Haliotis rufescens* fed with blades of the seaweed *Macrocystis pyrifera* epiphytized with a film of the benthic diatom *Navicula incerta*. Five treatments were used as food for juvenile abalone: (1) blades of *M. pyrifera* washed for 10 min with fresh water to remove natural epiphytes; (2) blades of *M. pyrifera* without natural epiphytes and colonized with *N. incerta* at an initial density of approximately 500 cells mm⁻²; (3) blades of *M. pyrifera* with natural epiphytes; (4) blades of *M. pyrifera* with natural epiphytes and colonized with *N. incerta*; and (5) batch cultures of *N. incerta* without blades. All treatments were kept on 18-L plastic buckets with 10 L of "f/2" medium at 16°C and 100 $\mu\text{Em}^{-2}\text{s}^{-1}$ of continuous light. All treatments were kept under culture conditions for 2 days. Plastic buckets of 18 L filled with 10 L of filtered seawater at 16°C was used for the bioassay with the red abalone. The five feeding treatments were provided *ad libitum* to juvenile abalone and were removed after 4 days. The water exchange rate in the buckets was ~300% per day. Every 15 days, and for a period of 90 days, weight and growth (as shell length and width) were measured. At the end of the experiment mortality was determined. The results showed that the highest growth rates and weight were observed in treatments 2, 4, and 5 whereas the lowest growth rates and weight were observed in treatments 1 and 3. The highest average survival was recorded in treatment 5 (52.17%), whereas the lowest was observed in treatment 2 (34.20%). This study showed that the enrichment of seaweed blades improves growth and survival in abalone and thus has potential to be used in abalone farms to enhance productivity.

KEY WORDS: benthic diatoms, epiphytes, seaweed, *Haliotis rufescens*, *Navicula incerta*, *Macrocystis pyrifera*

INTRODUCTION

Commercial abalone culture in México and California has traditionally been based on the supply of benthic diatoms as food for abalone postlarvae. When juvenile abalone reach a size of approximately 10 mm, the type of nourishment changes from benthic diatoms to seaweed. The main seaweed species used as food for juvenile and adult abalone are *Macrocystis pyrifera*, *Nereocystis luetkeana*, and *Egregia menziesii*, these and other brown seaweeds of the North Pacific coasts are abundant and easy to harvest from coastal waters (Hahn 1989, Buchal et al. 1998, McBride 1998).

The biochemical composition of seaweed varies among species and is related to the geographical zone, season, currents and wave exposure, nutrient availability, depth, temperature, and life stage of the algae (Cruz-Suárez et al. 2000). This variability in nutritional value produces heterogeneous growth rates and, in some cases, low growth in abalone (Bautista-Teruel & Millamena 1999).

The biochemical composition of the seaweed *Macrocystis pyrifera* is poor, ranging from 5% to 12% proteins, 0.5% to 1% lipids, and 46% to 50% carbohydrates (Cruz-Suárez et al. 2000). Benthic diatoms have a much better biochemical composition, 22% to 36% proteins, 13% to 58% lipids, and 5% to 23% carbohydrates (Renaud et al. 1999, Simental-Trinidad et al. 2001).

When the food for juvenile abalone is changed from benthic diatoms to seaweed it is believed to reduce growth and survival, and may be a factor involved in high mortalities of the organisms under culture conditions. The biochemical composition of seaweed can be enhanced by the performance of a selective colonization over the surface of the blade with benthic diatoms. The aim of this work is to improve the growth rate and survival of juvenile red abalone (*Haliotis rufescens*) by enhancing the biochemical com-

position of its food (*Macrocystis pyrifera*) with a selective epiphytization of the seaweed blades with the benthic diatom *Navicula incerta*.

MATERIALS AND METHODS

Blades of the seaweed *Macrocystis pyrifera* (excluding pneumatocysts) and cultures of the benthic diatom *Navicula incerta* were used in this work. The seaweed *M. pyrifera* was harvested every 4 days from a mantle located at Punta Morro (31°51'30"North, 116°38'38"West), on the coastal waters of Bahía Todos Santos, Baja California (BC), México. Every 4 days, from August to November 2002, seaweed was harvested at a depth of 2 m from the apical blades. The benthic diatom *N. incerta* was isolated from the same location by the staff of Universidad Autónoma de Baja California, and was also obtained by donation from the abalone farm "Abulones Cultivados" Ejido Eréndira, BC, México.

This study used 150-day-old juveniles of red abalone *Haliotis rufescens*, which were obtained from the earlier mentioned abalone farm. The abalone were selected to provide a group with an average shell length of 3.67 mm \pm 0.32 and weight of 0.0055 g \pm 0.0015. Organisms were acclimated to the experimental conditions for a period of 1 wk and fed *M. pyrifera ad libitum*.

Five treatments were used to provide food to juvenile abalone: (1) blades of *M. pyrifera* without natural epiphytes; (2) blades of *M. pyrifera* without natural epiphytes and colonized with *N. incerta*; (3) blades of *M. pyrifera* with natural epiphytes; (4) blades of *M. pyrifera* with natural epiphytes and colonized with *N. incerta*; and (5) batch cultures of *N. incerta* without blades.

All treatments were performed by triplicate and kept under controlled light irradiance (100 $\mu\text{Em}^{-2}\text{s}^{-1}$). The temperature for the experimental containers was maintained at 16°C by using an electronic controller.

*Corresponding author. E-mail: psanchez@cicese.mx

The seawater used for all experiments was passed through sand and cartridge filters of 10 μm and 5 μm , and finally irradiated with ultraviolet light. Cultures of *N. incerta* were prepared for inoculum in "f/2" medium (Guillard & Ryther 1962) in progressive volumes of 10 mL, 150 mL, 900 mL, and 10 L. The strain was acclimated to the culture conditions of temperature and continuous light previously described. For the production of the 10-L inoculum used in the bioassay with juvenile abalone, circular 18-L white plastic containers (30 \times 35 cm) with a lexon acrylic lid at the top were used (Simental-Trinidad et al. 2001). An initial density of 150 organisms per container was used in the bioassay. Seawater was exchanged at approximately 300% per day.

For treatment 1, blades of *M. pyrifera* were washed with freshwater for 10 min to remove all the natural epiphytes. After the bath, sets of two blades were placed in the plastic container with 10 L of "f/2" medium. For treatment 2, blades of seaweed were treated similarly to those in treatment 1, and were inoculated with a 4-day-old culture of *N. incerta* at an initial density of 50,000 cells mL^{-1} (obtaining approximately 500 cells mm^{-2} on the blade surface). For treatment 3, the seaweed blades were placed into the containers without any previous washing or inoculation. For treatment 4, the blades of *M. pyrifera* without previous washing were inoculated with *N. incerta*, similarly to the process described in treatment 2. Treatment 5 was a monospecific culture of *N. incerta* with the same cell density as the one used in treatments 2 and 4.

Two days after the setting of the 5 treatments, all were given as food to the abalone *ad libitum* and left for 4 days, after that time, excess food was removed and new treatments were added to the containers.

Every 15 days, and for a period of 90 days, weight and shell length were obtained by randomly sampling 15 organisms. Weight was measured with an electronic scale and shell length was measured by using a calibrated compound microscope. At the end of the experiment, survival of juvenile abalone was determined. Daily growth rates, in terms of weight (DGw) and shell length (DGsl), were evaluated as described by Capinpin & Corre (1996). Growth rate was calculated as follows:

$$\text{DGw } (\mu\text{g per day}) = \text{Gw}/n;$$

and

$$\text{DGsl } (\mu\text{m per day}) = \text{Gsl}/n;$$

where Gw is the increase in weight (μg), Gsl is the increase in shell length (μm) and n are the days of rearing.

To determine if there were significant differences in weight through time as a result of different treatments, a covariance statistical analysis was used. The same analysis was performed to determine differences in shell length, and shell width. To determine if there were significant differences in growth rates, a 1-way Anova was used. When differences were detected, a Tukey *a posteriori* test was performed. All the differences were evaluated at $\alpha = 0.05$. For all statistical analysis, the software Statistica 5.0 for Windows was used.

RESULTS

Significant differences in weight among treatments were detected ($F = 75.83$, $P < 0.00$) (Fig. 1). The highest weights were detected in treatments 2 ($0.100 \text{ g} \pm 0.005 \text{ SE}$) and 5 ($0.080 \text{ g} \pm 0.004$). The lowest abalone weights were obtained on treatments 1 ($0.046 \text{ g} \pm 0.003 \text{ SE}$) and 3 ($0.039 \text{ g} \pm 0.001 \text{ SE}$).

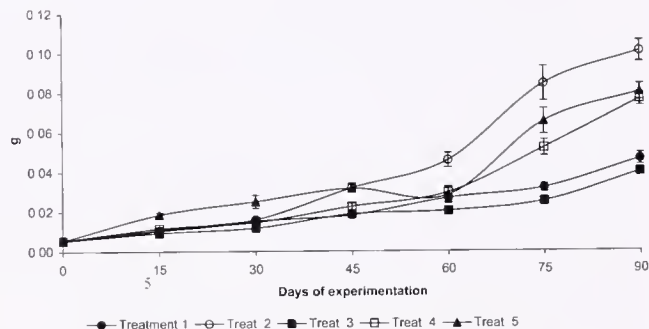


Figure 1. Mean weight of 150-day-old red abalone *Haliotis rufescens* fed with different seaweed diets. Treatments were: (1) *Macrocystis pyrifera* without natural epiphytes; (2) *M. pyrifera* without natural epiphytes and with a layer of *Navicula incerta*; (3) *M. pyrifera* with natural epiphytes; (4) *M. pyrifera* with natural epiphytes and with a layer of *N. incerta*, and (5) Culture of *N. incerta* without blades. Vertical bars indicate standard error.

Shell length showed significant differences ($F = 132.03$, $P < 0.00$) (Fig. 2). The highest shell lengths were detected on treatments 2 ($9.06 \text{ mm} \pm 0.09 \text{ SE}$) and 5 ($8.81 \text{ mm} \pm 0.14 \text{ SE}$). The lowest shell lengths were detected on treatments 1 ($6.76 \text{ mm} \pm 0.09 \text{ SE}$) and 3 ($8.20 \text{ mm} \pm 0.14 \text{ SE}$).

Significant differences were obtained for shell width ($F = 46.06$, $P < 0.00$) (Fig. 3). A maximum value of shell width was recorded on treatment 2 ($6.17 \text{ mm} \pm 0.010 \text{ SE}$). The lowest values were detected on treatments 1 ($4.57 \text{ mm} \pm 0.07 \text{ SE}$) and 5 ($5.66 \text{ mm} \pm 0.08 \text{ SE}$).

The average daily growth rate for the 5 treatments in terms of weight and shell length of abalone for 0–30 days, 30–60 days, and 60–90 days are shown in Table 1. DGw showed significant differences ($F = 23.4$, $P < 0.00$) on days 30–60 and 60–90. Abalone fed on treatments 2, 4, and 5 produced the highest growth rates in terms of weight. Regarding growth rate expressed as DGsl, a decrease was observed from day 30 until the end of the experiment. There were significant differences only during the 30–60 day period, when abalone were fed on treatments 2, 4, and 5 and where the highest growth rates in terms of shell length were recorded.

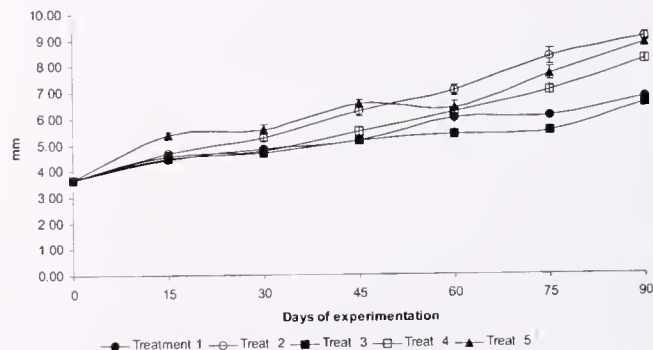


Figure 2. Mean shell length of 150-day-old red abalone *Haliotis rufescens* fed with different seaweed diets. Treatments were: (1) *Macrocystis pyrifera* without natural epiphytes; (2) *M. pyrifera* without natural epiphytes and with a layer of *Navicula incerta*; (3) *M. pyrifera* with natural epiphytes; (4) *M. pyrifera* with natural epiphytes and with a layer of *N. incerta*; and (5) Culture of *N. incerta* without blades. Vertical bars indicate standard error.

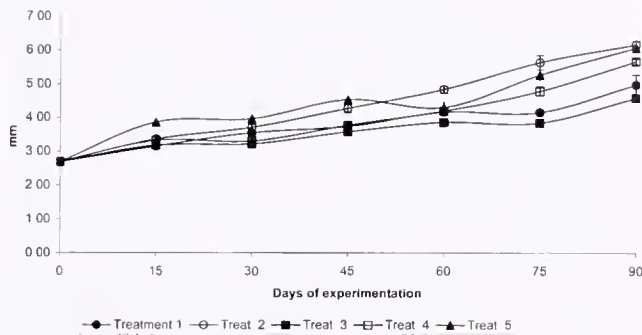


Figure 3. Mean shell width of 150-day-old red abalone *Haliotis rufescens* fed with different seaweed diets. Treatments were: (1) *Macrocystis pyrifera* without natural epiphytes; (2) *M. pyrifera* without natural epiphytes and with a layer of *Navicula incerta*; (3) *M. pyrifera* with natural epiphytes; (4) *M. pyrifera* with natural epiphytes and with a layer of *N. incerta*; and (5) Culture of *N. incerta* without blades. Vertical bars indicate standard error.

Minimum and maximum survival is presented in Table 2. There were significant differences between repetitions, with a minimum survival value recorded on treatment 2 (9.56%) and a maximum survival value on treatment 4 (82.60%).

DISCUSSION

In natural populations, abalone are nonselective feeders (Leighton & Boolootian 1963). In Mexican farms, where commercial abalone production is performed, food supply is based on seaweed instead of formulated diets. The seaweed is used mainly because of its availability, easy harvesting methods, and low cost. The main species used are *Macrocystis pyrifera* and *Gracilaria* spp. (Cabal-Miranda pers. com. 2003). Juvenile begin to eat macroalgae at about 10 mm in length and will eat from 10% to 30% of their whole-body wet weight in algae each day. The high feeding rate of macroalgae is due to the high water content and relative low protein content of fresh macroalgae (Hahn 1989).

Usually, the protein content of benthic diatoms, such as *Navicula* sp. under culture conditions, varies according to the age and culture conditions from 20% to 30%; whereas carbohydrate content ranges from 10% to 15%, and lipid content ranges between 15% to 20% (Flores-Vergara 1998, Correa-Reyes et al. 2001, Car-

bajal-Miranda 2002, Simental-Trinidad et al. 2001, Simental & Sánchez-Saavedra 2003).

In Mexican abalone farms, the change in nourishment, from microalgae to seaweed, is initiated when juvenile abalone are 3 mo old (Vázquez-Moreno pers. com. 2002) or when they are close to 10 mm in length. The daily seaweed portion that juvenile abalone receive ranges from 10% to 30% of their whole-body wet weight (Hahn 1989). According to Kawamura et al. (2001), the structure of the radula and digestive abilities for the use of seaweed as food is ready at 2–4 mm shell length in *H. discus hannai*; thus, the juvenile *H. rufescens* used in this experiment (3.67 ± 0.32 mm) were fully able to feed on seaweed.

In an experiment performed with deep-sea water and a continuous and simultaneous culture of the benthic diatom *Nitzschia* sp., used as food for 7-mo-old juvenile abalone *Haliotis sieboldi* ($12.4 \text{ mm} \pm 0.2 \text{ Sd.}$), daily growth rate of the organisms was 50 to $110 \mu\text{m day}^{-1}$. Such results show that continuous cultivation of *Navicula* sp. with deep seawater promotes growth of juvenile abalone, without the need for providing supplementary seaweed or artificial diets (Fukami et al. 1997). The best growth rate in length and weight of *H. rufescens* was obtained from treatments with selective epiphytation or enriched with the benthic diatom *Navicula incerta*. Juvenile abalone retains the ability to digest diatoms after developing the ability to digest macroalgae. A combined diet of benthic diatoms and macroalgae provides greater nourishment and faster growth rate.

A common practice on abalone farms is the washing of the seaweed blades to avoid the introduction of other algae, microorganisms, and bacteria. However, research has shown that the epiphytes can contribute to an increase in the chemical composition of seaweed (Simental-Trinidad 2003). When abalone were fed with blades of *Laminaria religiosa* with bryozoa (*Membranipora membranacea*) on the surface, they grew faster than those fed with the same species without bryozoa. These are evidences that bryozoa probably supply complementary nourishment to the abalone (Uki 1981). Research suggests that abalone also can use bacteria, yeast, and other micro-organisms associated with diatoms as food (McBride & Conte 2001).

Benthic diatoms are the main diet for small abalone (Voltolina 1985, 1994, Daume et al. 1997, Daume et al. 1999, 2000, Kawamura 1996, Kawamura et al. 1998a, Kawamura et al. 1998b; Roberts 2000; Searcy-Bernal et al. 2000). Some diatoms in feces were observed to have cytoplasm and nuclear material and were there-

TABLE 1.

Average daily growth rates in terms of weight (DGW) and shell length (DGL) of red abalone *Haliotis rufescens* fed with different treatments* using the seaweed *Macrocystis pyrifera* and the benthic diatom *Navicula incerta*.

Treatment*	DGW ($\mu\text{g day}^{-1}$)			DGL ($\mu\text{m day}^{-1}$)		
	Day 0–30	Day 30–60	Day 60–90	Day 0–30	Day 30–60	Day 60–90
1	323.53 a (7.41)	189.07 ab (53.12)	234.81 a (95.48)	38.47 a (5.88)	19.17 ab (2.13)	9.15 a (4.10)
2	338.8942 a (67.66)	503.39 b (90.10)	824.53 b (203.84)	52.24 a (9.27)	29.94 b (5.06)	25.01 a (3.90)
3	206.50 a (57.97)	142.64 a (8.28)	208.43 a (87.05)	33.36 a (9.08)	11.92 a (3.20)	12.09 a (6.01)
4	298.97 a (66.07)	249.12 ab (65.38)	577.34 ab (88.16)	35.68 a (1.78)	24.33 ab (2.95)	24.39 a (2.88)
5	645.91 a (76.93)	208.06 a (51.1)	579.85 ab (194.62)	63.32 a (5.66)	15.29 ab (4.58)	27.14 a (7.19)

* Treatments were: (1) *Macrocystis pyrifera* without natural epiphytes; (2) *M. pyrifera* without natural epiphytes and with a layer of *Navicula incerta*; (3) *M. pyrifera* with natural epiphytes; (4) *M. pyrifera* with natural epiphytes and with a layer of *N. incerta*; (5) Culture of *N. incerta* without blades. The standard error is included in parenthesis.

The different letters on the side of the quantities indicate significant differences (1-way Anova and Tukey *a posteriori* test, $\alpha = 0.05$): a < b.

TABLE 2.

Average survival and minimum and maximum values due to replicates (expressed in percentages) of juvenile red abalone *Haliotis rufescens*, after twelve weeks of feeding with different treatments* using the seaweed *Macrocystis pyrifera* and the benthic diatom *Navicula incerta*.

Treatment*	Average (%)	Survival	
		Maximum (%)	Minimum (%)
1	48.40	57.39	38.26
2	34.20	67.82	9.56
3	46.95	55.65	40.00
4	52.17	82.60	30.43
5	50.86	53.04	48.69

* Treatments were: (1) *M. pyrifera* without natural epiphytes; (2) *M. pyrifera* without natural epiphytes and with a layer of *N. incerta*; (3) *M. pyrifera* with natural epiphytes; (4) *M. pyrifera* with natural epiphytes and with a layer of *N. incerta*; (5) Culture of *N. incerta* without blades.

fore, assumed not to have been completely digested. In the gut content of *Haliotis asinina* collected from the natural environment, it was found that the main nourishment appears to be composed of benthic diatoms. Dominant genera in gut content were *Nitzschia*, *Amphora*, *Navicula*, and *Cocconeis*, which were the predominant floral elements in the local environment (Sawatpeera et al. 1998). Relatively few diatom strains are ruptured when eaten by abalone postlarvae. Postlarvae <1 mm in shell length can grow using extracellular foods such as mucus from adult abalone. Postlarvae >1 mm require the cell content of diatoms for rapid growth. The natural succession of diatom communities can be controlled, to some extent, by light and grazing pressure, thus, favoring useful diatoms (Roberts et al. 2000).

The nutritional value of monospecific cultures of eight benthic diatom species was determined for abalone postlarvae of *Haliotis rufescens*. The best growth rate, measured as shell length, was obtained when organisms were fed with *Amphiprora paludosa* var. *hyalina*, *Nitzschia thermalis* var. *minor*, and *Navicula incerta* (Correa-Reyes et al. 2001, Correa-Reyes 2002).

The growth rate obtained in this work varied from 9.15–63.32 $\mu\text{m day}^{-1}$. However, Kawamura et al. (1995) determined growth rates from 13.6–50.1 $\mu\text{m day}^{-1}$ in *H. discus hannai* fed with several benthic diatoms. Meanwhile, Neori et al. (2000) reported

growth rate values of 66.6 $\mu\text{m day}^{-1}$ in *H. discus hannai* fed with *Ulva lactuca* or *Gracilaria conferta*. In this experiment we obtained better growth rates when abalone were fed with blades of seaweed containing benthic diatoms. Another important consideration is that both species (*M. pyrifera* and *N. incerta*) are commonly used on abalone farms in México.

The use of seaweed as a food source on abalone farms has some other advantages due to the fact that they can also act as biologic filters and can be used as a coculture of abalone and seaweed (Evans & Langdon 2000). Thus, establishing the possibility for the use of systems with low water exchange or closed systems for the culture of abalone without the problem of losing stability of formulated diets, which in some cases lose as much as 24% of dry matter in their pellets (Guzmán & Viana 1998). Consequently, decreasing problems of waste removal and aeration, which are the main limiting factors, considered by many farmers, when using artificial diets (Fleming et al. 1996). Eutrophication by nitrogen can reduce the diversity of communities, and produce low water quality (Hillebrand & Sommer 2000). With seaweeds such as *Gracilaria* used as biofilters, 90% of the nitrogen present in the water can be removed (Ryther et al. 1982).

CONCLUSION

A significant increase in weight and shell length for juvenile abalone was determined over time when the organisms were fed with the algae *Macrocystis pyrifera*, selective epiphytes with cultures of the benthic diatom *Navicula incerta*, and with the monocultures of *N. incerta*. Growth rate and survival of juvenile abalone (*Haliotis rufescens*) can be increased by using the seaweed *Macrocystis pyrifera* and selective epiphytes with cultures of the benthic diatom *Navicula incerta* as feed.

ACKNOWLEDGMENTS

The authors thank M. Carbajal-Miranda, M. Pacheco-Vega, C. Cruz-Fraga and S. Fierro-Reséndiz for technical assistance, and L. Salinas-Flores and M. Segovia-Quintero for manuscript revision. The authors also thank Consejo Nacional de Ciencia y Tecnología (CONAC y T) for the Ph. D scholarship to the first author. This work was supported by economic grants of Centro de Investigación y de Educación Superior de Ensenada (CICESE, Project 6554) and Consejo Nacional de Ciencia y Tecnología (CONAC y T, Project 33016B).

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COMBINED EFFECTS OF LIGHT CONDITION (CONSTANT ILLUMINATION OR DARKNESS) AND DIATOM DENSITY ON POSTLARVAL SURVIVAL AND GROWTH OF THE ABALONE *HALIOTIS RUFESCENS*

ERÉNDIRA GORROSTIETA-HURTADO¹ AND RICARDO SEARCY-BERNAL^{2*}

¹Centro de Investigación Científica y de Educación Superior de Ensenada, Km 107 Carretera Tijuana-Ensenada, Ensenada, B. C. México, 22800; ²Instituto de Investigaciones Oceanológicas, Universidad Autónoma de Baja California, Km 103 Carretera Tijuana-Ensenada, Apartado Postal 453, Ensenada, B. C. México, 22860

ABSTRACT Abalone (*Haliotis* spp.) postlarvae are cultured in systems that provide natural or artificial light to promote the growth of benthic diatoms that are grazed by postlarvae. Larger abalones (>2 cm) grow better in dark conditions and the possibility that this is true for postlarvae is explored in this contribution. Two independent experiments with *Haliotis rufescens* postlarvae fed the diatom *Navicula incerta* were conducted in 10-mL vessels with daily water changes. Two factors were tested following split-plot experimental designs: six diatom densities (from 500–10,000 cells/mm²) and two light conditions (constant light at 19–33 $\mu\text{E}/\text{m}^2/\text{s}$ and darkness). Experimental units in darkness were kept inside black plastic bags but subjected to ~30 min of ambient light every day for maintenance. Food (*N. incerta*) was supplied as required to maintain diatom densities. The first experiment started with 14-day-old postlarvae and was conducted for 20 days; the second trial started with 2-day old postlarvae and was performed for 32 days. In general, postlarval growth increased as diatom density increased but stabilized at high densities (ca. >2,000 cell/mm²) and was significantly higher in darkness in both trials. In Experiment 1, average growth rate in darkness was 2.4 times higher than in light conditions (34.7 and 14.4 $\mu\text{m}/\text{d}$, respectively). In Experiment 2, average growth in darkness was 3.0 times higher than under constant illumination (14.4 and 4.8 $\mu\text{m}/\text{d}$, respectively). These results are discussed in terms of postlarval behavior and possible changes in the nutritional quality of diatom films. The potential implications for abalone culture are also addressed.

KEY WORDS: abalone postlarvae, survival, growth, darkness, diatom density

INTRODUCTION

The red abalone (*Haliotis rufescens*) is the largest abalone species in the world, reaching up to 28 cm in shell length (Hahn 1989, Leighton 2000). It represents 95% of the abalone culture in California (USA) (Leighton 2000) and is also the most important species cultured in Baja California, México (McBride 1998). Abalone culture is a high-risk activity and mortalities can reach up to 99% after 3 y (Hone et al. 1997). In the postlarval stage, which begins after settlement and metamorphosis, the highest mortality is usually reported (80% to 95%) (Searcy-Bernal 1996, Hone et al. 1997).

In abalone postlarvae, growth is influenced by several factors including type of food (Kawamura et al. 1998a, Roberts et al. 1999, Daume et al. 2000), quantity of food (Searcy-Bernal et al. 2001), starvation period (Roberts et al. 2001, Takami et al. 2000), temperature (Leighton 1974), and light intensity (Searcy-Bernal et al. 2003). For example, postlarvae of the blue abalone *Haliotis fulgens* grow better in low irradiances (6 $\mu\text{E}/\text{m}^2/\text{s}$) than in higher light intensities (24–75 $\mu\text{E}/\text{m}^2/\text{s}$), probably because of differences in ecologic conditions in the biofilms developed over culture surfaces (Searcy-Bernal et al. 2003). In the abalone farms of California and Baja California, postlarvae are usually maintained at irradiances from 3 to 200 $\mu\text{E}/\text{m}^2/\text{s}$ under natural photoperiods or continuous artificial light (Searcy-Bernal et al. 2003). Although the effect of photoperiod on postlarval growth is unknown, diatoms need light for their growth, which is important to maintain a good density of cells to feed the postlarvae.

Juvenile and adult abalones have a circadian rhythm; they are more active and feed at night (Hahn 1989, Leighton 2000). Some studies have shown increased growth rates when these gastropods

are cultured in darkness (Ebert & Houk 1984, Godoy-Corrales 1989). Research addressing this issue for postlarvae is lacking, although a preliminary study provided some evidence that postlarvae of *H. fulgens* increase their grazing rates at dusk (Vélez-Espino 1999).

Recent studies have shown that the quantity of food available for postlarvae is an important factor determining their growth. Grazing and growth rates of *H. fulgens* postlarvae increase as the density of the diatom *Navicula incerta* increases from 500 to 4,000 cells/mm² (Searcy-Bernal et al. 2001) but studies for other species are lacking.

The objective of this work is to evaluate the combined effect of continuous light or darkness conditions and different densities of the diatom *N. incerta* on the survival and growth of red abalone (*H. rufescens*) postlarvae.

MATERIALS AND METHODS

Abalone larvae were provided by the commercial farm Abulones Cultivados (Eréndira, B.C., México) on March and May 2002, transported to the Instituto de Investigaciones Oceanológicas (IIO) laboratory and used in Experiments 1 and 2, respectively. Competent larvae were induced to settle by adding 1.5 μM of gamma-aminobutyric acid (GABA) (Searcy-Bernal & Anguiano-Beltrán 1998).

Monocultures of *N. incerta*, a benthic diatom widely used to feed abalone postlarvae, were provided by the IIO Microalgae Laboratory. Diatoms were inoculated in 250 mL Erlenmeyer flasks containing 150 mL of f/2 medium (Guillard 1975) and cultured under constant temperature ($17 \pm 1^\circ\text{C}$) and illumination (37 $\mu\text{E}/\text{m}^2/\text{s}$, provided by fluorescent day-light lamps). After 3–4 days of culture, the flasks were immersed in an ultrasound bath (Fisher Scientific FS6) for 3 min to detach the diatoms, which were

*Corresponding author. E-mail: rsearcy@uabc.mx

counted using a hemacytometer to estimate cellular density and to calculate diatom densities to be used in the experiments.

Experiment 1

Settlement was carried out in plastic containers ($57 \times 37 \times 13$ cm) containing 5 L of filtered ($1 \mu\text{m}$) and UV-irradiated seawater. After metamorphosis, postlarvae were fed with *N. incerta* (250 cells/ mm^2) and cultured in these containers for 14 days. Seawater was changed every 2 days and diatoms were added every week.

The experiment was conducted in six-well tissue culture plates (Falcon 3046, 9.45 cm^2 bottom area) with 10 mL of $1 \mu\text{m}$ -filtered UV-irradiated seawater per well. Water changes were performed daily.

Two light conditions (light and dark) and six diatom densities were tested following a split-plot experimental design (Steel et al. 1997). Three culture plates (whole units) were placed inside thick black polyethylene bags to obtain dark conditions, and three plates were in continuous light of between 24 and $33 \mu\text{E}/\text{m}^2/\text{s}$ (fluorescent day-light lamps). Six densities of *N. incerta* (250, 500, 750, 1,000, 2,000, and 3,000 cells/ mm^2) were randomly inoculated in the wells (subunits) of each plate. After 1 day, ten 14-day-old postlarvae ($480 \mu\text{m}$ average shell length) were placed in each well. Water changes were performed daily. The culture plates in the dark treatment were exposed daily to light for ~ 30 min during water changes.

After a week in these food densities all wells were cleaned of diatoms and feces with a brush and rinsed two or three times with seawater. Then, the wells were reinoculated with higher densities of *N. incerta* (500, 1,000, 2,000, 4,000, 8,000, and 10,000 cells/ mm^2). These final diatom densities were not applied during the first days of the experiment, because previous experience had shown deleterious effects of high densities on early postlarvae. Every 4 days, the wells were cleaned as described earlier and were reinoculated with these final diatom densities of *N. incerta*.

Experiment 2

The same experimental design and vessels were used, but some different procedures were followed. Competent larvae (13–15) were placed and settled with GABA in the wells of culture plates. Irradiance of the culture plates under continuous light was between 19 and $21 \mu\text{E}/\text{m}^2/\text{s}$. Sea water quality ($1 \mu\text{m}$ -filtered, UV-irradiated) was further improved by sterilization (in an autoclave). Two days after metamorphosis induction postlarvae were fed with 6 densities of *N. incerta* (250, 500, 750, 1000, 2000, and 3000

cells/ mm^2). Every 4 days, the wells were cleaned and rinsed as described in Experiment 1 and reinoculated with these initial diatom densities. After 16 days (when postlarvae were 18-days old), reinoculation densities were increased to 500, 1,000, 2,000, 4,000, 8,000, and 10,000 cells/ mm^2 . These differences between experiments are summarized in Table 1.

Both experiments were conducted at $17^\circ\text{C} \pm 1^\circ\text{C}$. Survival was determined by counting live postlarvae in each well. To determine shell length and growth rates, all surviving postlarvae were video-recorded with a high-resolution camera (Sony SSC-C374) in an inverted microscope (Meiji Techno). Images were digitalized in a computer and measurements of shell length were performed with the program Scion Image (4.0.2). Survival and shell lengths were evaluated at days 9 and 20 in Experiment 1, and at days 10, 18, and 32 in Experiment 2 (in this trial day 0 corresponded to the day of the first diatom addition, i.e., 2 days after metamorphosis induction).

Statistical analysis was performed using the program JMP (version 3.2.6, SAS Institute Inc.). Split-Plot analyses of variance (ANOVA) (Steel et al. 1997) were used to evaluate the effect of diatom densities, light condition and their interaction on postlarval survival, shell length, and growth rates. When significant interactions were found, separate ANOVAs and multiple comparisons (LSD tests) were performed to evaluate the effect of diatom density within each light condition. Percent survival data were transformed before analysis (arcsine square root). A value of $\alpha = 0.05$ was chosen as the significance level.

RESULTS

Survival

In Experiment 1, survival was high ($>80\%$) in all treatments during the experimental period of 20 days (Fig. 1) and there were no significant over-all effects of diatom density or light condition, although in the first 9 days a significant interaction was found (Table 2) reflecting a different effect of one factor in levels of the other. Separate ANOVA tests within each light condition showed a significant effect of diatom density only in the dark ($F = 3.71$, $P = 0.029$) where a significantly lower survival in 2,000 cells/ mm^2 was detected (LSD tests, Fig. 1).

In Experiment 2, survival after the 32-day experimental period was below 70% (Fig. 2). Treatment effects were not significant during the first 10 days; however, after 18 days a significant interaction was detected (Table 3). Survival in the dark was lowest

TABLE 1.

Differences between experiments. Both were conducted at $17 \pm 1^\circ\text{C}$ in sterile six-well tissue culture plates (10–15 postlarvae per well). Seawater was changed daily and reinoculations of diatoms at the experimental densities were performed every 4 days.

Procedure	Experiment 1 (March 2002)	Experiment 2 (May 2002)
Settlement (GABA, $1.5 \mu\text{M}$)	In 51 containers	In wells
Seawater quality	$1 \mu\text{m}$ -filtered, UV-irradiated	$1 \mu\text{m}$ -filtered, UV-irradiated, sterilized
Irradiance at continuous light treatment	$24\text{--}33 \mu\text{E}/\text{m}^2/\text{s}$	$19\text{--}21 \mu\text{E}/\text{m}^2/\text{s}$
Initial age of postlarvae (after metamorphosis induction)	14 d	2 d
Low range of diatom densities (250–3,000 cells/ mm^2)	At postlarval ages: 14–21 d	At postlarval ages: 2–18 d
High range of diatom densities (500–10,000 cells/ mm^2)	At postlarval ages: 21–34 d	At postlarval ages: 18–34 d

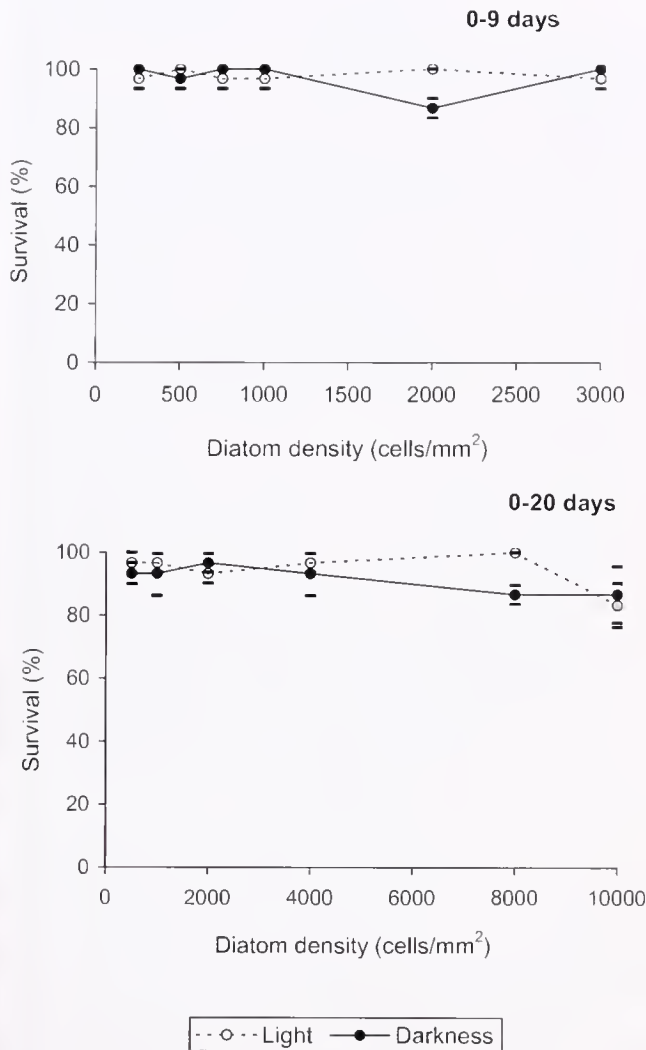


Figure 1. Survival of *H. rufescens* postlarvae (mean \pm SE) in different light conditions and diatom densities in Experiment 1 during the first evaluation period (0–9 days) and the complete experimental period (0–20 days). Initial postlarval age was 14 days.

(45%) at the lowest diatom density (500 cells/mm²), increased as density increased up to 2,000 cells/mm² and remained more or less constant at higher densities (ca. 75%). In the light treatment survival was higher at intermediate diatom densities up to 8,000 cells/mm² (ca. 60%) but decreased drastically to 24% at the highest

density (10,000 cells/mm²) (Fig. 2). This survival was significantly lower than in the dark (76%) at the same diatom density ($F = 23.27$, $P = 0.008$). Similar survival patterns were observed at the final evaluation (32 days) (Fig. 2) when significant diatom and interaction effects were detected (Table 3). This interaction implied a significant effect of diatom density only in the dark ($F = 3.34$, $P = 0.040$), with the lowest survival (6%) at the lowest diatom density and more or less constant survival (ca. 60%) at densities above 2,000 cells/mm² (LSD tests). In this experiment survival was higher in dark conditions for most diatom densities. At the highest density (10,000 cells/mm²), final survival averages were 66% and 13% for the dark and light treatments, respectively, and this difference was significant ($F = 24.99$, $P = 0.007$).

Shell Length and Growth Rates

In both experiments, initial postlarval shell lengths were not significantly different among treatments. However, postlarval lengths and growth rates were significantly higher in darkness conditions at the end of the experimental periods.

In Experiment 1, final average shell lengths between 1,250 and 1,290 μm were obtained at the four higher diatom densities under darkness, corresponding to growth rates of 37–40 $\mu\text{m}/\text{d}$. In the light treatment the maximum length attained was 792 μm at 4,000 cells/mm² (15 $\mu\text{m}/\text{d}$) (Fig. 3, Fig. 4). This effect of light condition was already significant since the first evaluation (0–9 days, postlarval ages: 14–23 days) (Table 2). Diatom density did not have a significant impact on postlarval growth during the first 9 days but significant treatment and interaction effects were detected thereafter (Fig. 4, Table 2). At the end of the 20-day experimental period, diatom effects were significant only in the dark ($F = 23.60$, $P < 0.001$), showing an asymptotic behavior of growth after 2,000 cells/mm², whereas the slow growth (12–16 $\mu\text{m}/\text{d}$) in the light treatment was unaffected by diatom density ($F = 2.14$, $P = 0.130$) (Fig. 4).

In Experiment 2, the effect of light condition on postlarval growth was significant at the first evaluation and thereafter (Fig. 5, Table 3). A maximum final length of 1,084 μm (corresponding to a growth rate of 24 $\mu\text{m}/\text{d}$) was obtained in darkness at the highest diatom density (10,000 cells/mm²) and shell length decreased as diatom density decreased. In the light treatment a maximum final length of 546 μm was observed at 8,000 cells/mm² (7 $\mu\text{m}/\text{d}$) (Fig. 5, Fig. 6). Diatom density and interaction effects on growth rate were also significant from the first evaluation onwards (Table 3). At the end of the 32-day experimental period diatom effects were significant in both light conditions ($F = 11.96$, $P < 0.001$ and $F =$

TABLE 2.
Results of split-plot ANOVAS for survival and growth rate of *H. rufescens* postlarvae in Experiment 1.

	Days in Treatment	Postlarval Age (d)	Light Condition (df = 1, 4)		Diatom Density (df = 5, 20)		Interaction (df = 5, 20)	
			F	P	F	P	F	P
Survival	9	23	0.025	0.882	1.272	0.314	4.307	0.008
	20	34	3.361	0.141	0.960	0.465	1.039	0.422
Growth rate	0–9	14–23	614.57	2×10^{-5}	0.82	0.547	2.03	0.118
	9–20	23–34	131.03	3×10^{-4}	51.38	9×10^{-11}	46.71	2×10^{-10}
	0–20	14–34	278.08	8×10^{-5}	20.78	3×10^{-7}	17.23	1×10^{-6}

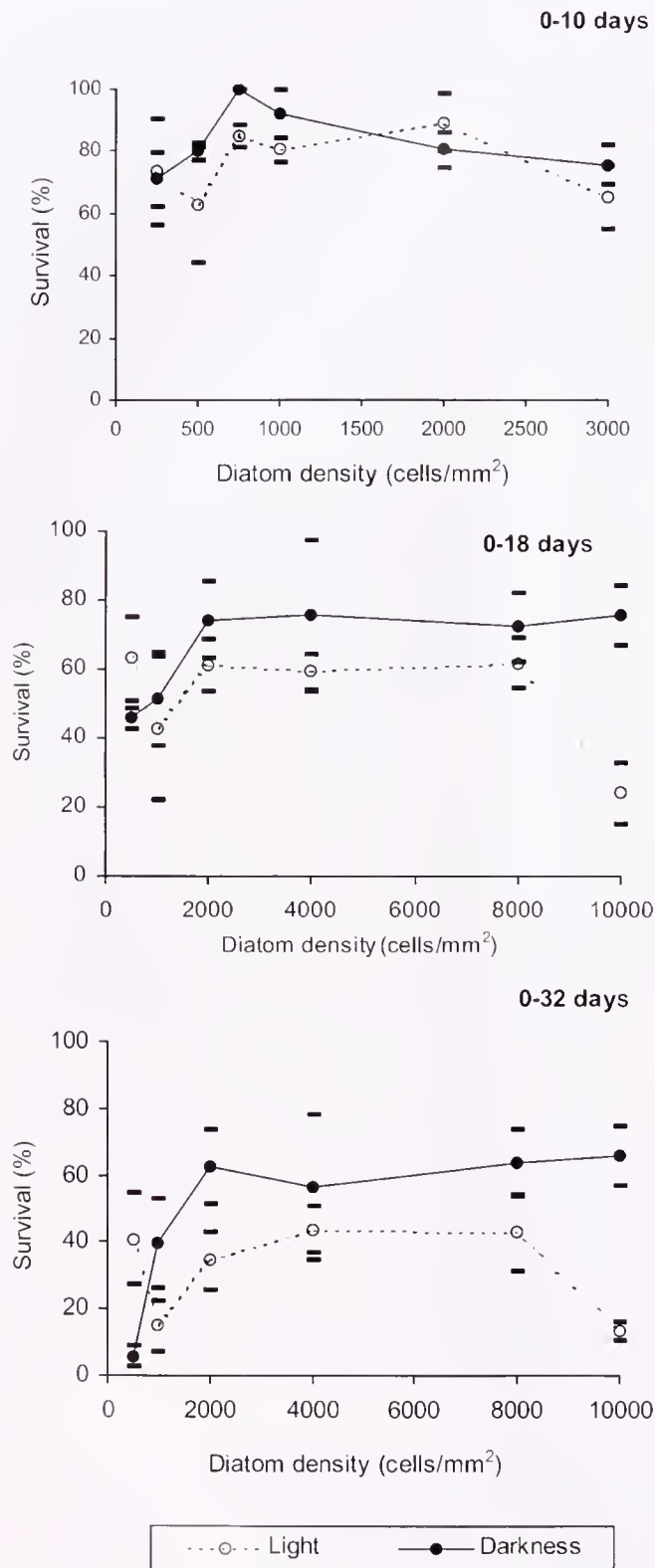


Figure 2. Survival of *H. rufescens* postlarvae (mean \pm SE) in different light conditions and diatom densities in Experiment 2 during three evaluation periods. Initial postlarval age was 2 days.

6.16, $P = 0.005$ for darkness and light, respectively), following a similar pattern than in Experiment 1 with a marked suppression of growth below 2,000 cells/mm² in the dark and little effect of diatom density in the light treatment (Fig. 6).

DISCUSSION

Effects of Light or Dark Condition

Although a significant effect of light condition on postlarval survival of *H. rufescens* was not detected, the survival pattern in Experiment 2 (Fig. 2) suggests adverse effects of light at high diatom densities. Survival also decreased slightly at the highest diatom density in light conditions in Experiment 1 (Fig. 1) but this effect was not as sharp as in Experiment 2 probably because postlarvae were older and presumably more resistant to environmental stress. A similar pattern was observed in an experiment with *H. fulgens* where postlarval survival after 1 mo was 90% at 6 $\mu\text{E}/\text{m}^2/\text{s}$ and only 3% at 47 $\mu\text{E}/\text{m}^2/\text{s}$ (Searcy-Bernal et al. 2003). This might be related to oxygen supersaturation conditions in the boundary layer (postlarval microhabitat) at high diatom densities when light is available for photosynthesis.

Oxygen conditions in the boundary layers over diatom films can change dramatically according to light conditions. Searcy-Bernal (1996) detected 140% and 50% oxygen saturation over *Nitzschia* sp. films at 59 and 4 $\mu\text{E}/\text{m}^2/\text{s}$, respectively, and Roberts et al. (2000) report oxygen saturation values of 400% and 60% over *Achnanthes longipes* films under light and dark conditions. Although preliminary evidence suggests the abalone postlarvae can tolerate high oxygen concentrations (Loipersberger 1996), the concentrations reached at the highest diatom density in Experiment 2 under light conditions, might have been above the tolerance limits of early *H. rufescens* postlarvae.

On the other hand, the high survival and growth of postlarvae at high diatom densities under darkness, suggest that they can tolerate very low oxygen concentration (expected under such conditions because of oxygen consumption by diatoms) without adverse effects.

The effect of light conditions on postlarval growth was dramatic in both experiments. In Experiment 1, the average growth rate (over all diatom densities) in darkness was 2.4 higher than in light conditions (34.7 and 14.4 $\mu\text{m}/\text{d}$, respectively). In Experiment 2, average growth in darkness was 3.0 times higher than under constant illumination (14.4 and 4.8 $\mu\text{m}/\text{d}$, respectively). Searcy-Bernal et al. (2003) also report a positive effect of low irradiances, because postlarval growth of *H. fulgens* postlarvae was higher at 6 $\mu\text{E}/\text{m}^2/\text{s}$ than at 47 $\mu\text{E}/\text{m}^2/\text{s}$ (37 and 21 $\mu\text{m}/\text{d}$, respectively). This might be explained by differences in postlarval feeding behavior and/or ecological conditions of biofilms.

Adults and juvenile abalones have nocturnal feeding habits. Commonly during daylight hours abalones remain relatively quiet and tend to aggregate in darkened locations. Grazing activity begins about half hour before sunset and continues throughout the night to satiation (Leighton 2000). Several studies have shown an increase in feeding and growth rates when abalones are cultured in darkness (Ebert & Houk 1984, Godoy-Corrales 1989, Kim et al. 1997).

This pattern has not been documented for postlarvae, which are generally assumed to feed all day because they do not seem to have the same negative phototactic behavior as juveniles and adults. However, this may not be true and nocturnal feeding habits might be established during the postlarval stage. Vélez-Espino (1999) evaluated grazing rates of *H. fulgens* postlarvae during a 24-h cycle and found increased feeding about an hour before sunset in 15- and 30-day-old postlarvae, which is consistent with the feeding behavior of larger abalones. In the experiments reported here, a higher fecal production was qualitatively observed in the darkness

TABLE 3.

Results of split-plot ANOVAS for survival and growth rate of *H. rufescens* postlarvae in Experiment 2.

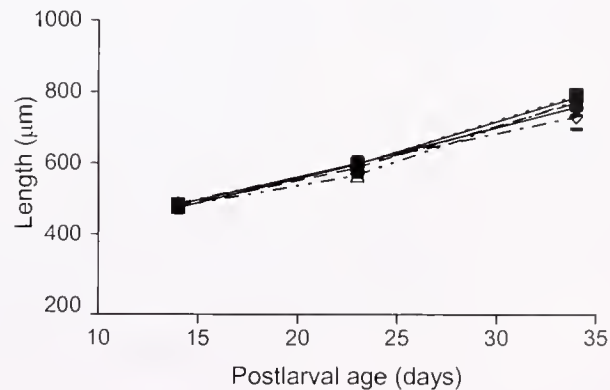
	Days in Treatment	Postlarval Age (d)	Light Condition (df = 1, 4)		Diatom Density (df = 5, 20)		Interaction (df = 5, 20)	
			F	P	F	P	F	P
Survival	10	12	0.900	0.396	2.552	0.061	1.401	0.266
	18	20	3.029	0.157	1.957	0.129	2.568	0.060
	32	34	3.833	0.122	3.438	0.021	4.55	0.006
Growth rate	0-10	2-12	25.37	0.007	5.97	0.001	4.10	0.01
	10-18	12-20	26.46	0.007	21.39	2×10^{-7}	8.80	1×10^{-4}
	18-32	20-34	29.24	0.006	7.34	4×10^{-4}	2.61	0.056
	0-32	2-34	91.66	6×10^{-4}	11.79	2×10^{-5}	4.53	0.006

treatments during the cleaning procedures, which is consistent with the hypothesis of increased feeding under dark conditions, which would in turn increase postlarval growth.

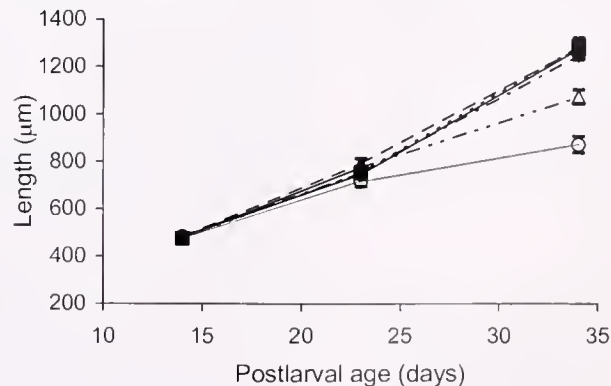
Light conditions might have an impact on ecologic conditions or nutritional value of the biofilm affecting postlarval growth. An important nutritional source for abalone postlarvae is provided by the mucus produced by diatoms and bacteria in the biofilm (Kawamura & Takami 1995, Kawamura et al. 1998a). This mucus is

composed of extracellular polymeric substances (EPS) (Decho 1990), which are produced at a higher rate in darkness than under light conditions by the benthic diatom *Navicula perminuta* (Smith & Underwood 2000). This suggests that an increased nutritional value of diatom films under darkness might help to explain the higher growth rates of abalone postlarvae. On the other hand, ecologic conditions in the boundary layer in the light treatment might have limited postlarval growth (Searcy-Bernal 1996).

Exp. 1: Light



Exp. 1: Darkness



—○— 500 —△— 1000 —◇— 2000 —■— 4000 —▲— 8000 —●— 10000 cells/cm²

Figure 3. Shell length (mean \pm SE) of *H. rufescens* postlarvae at different ages in the experimental light conditions and densities of *N. incerta* in Experiment 1.

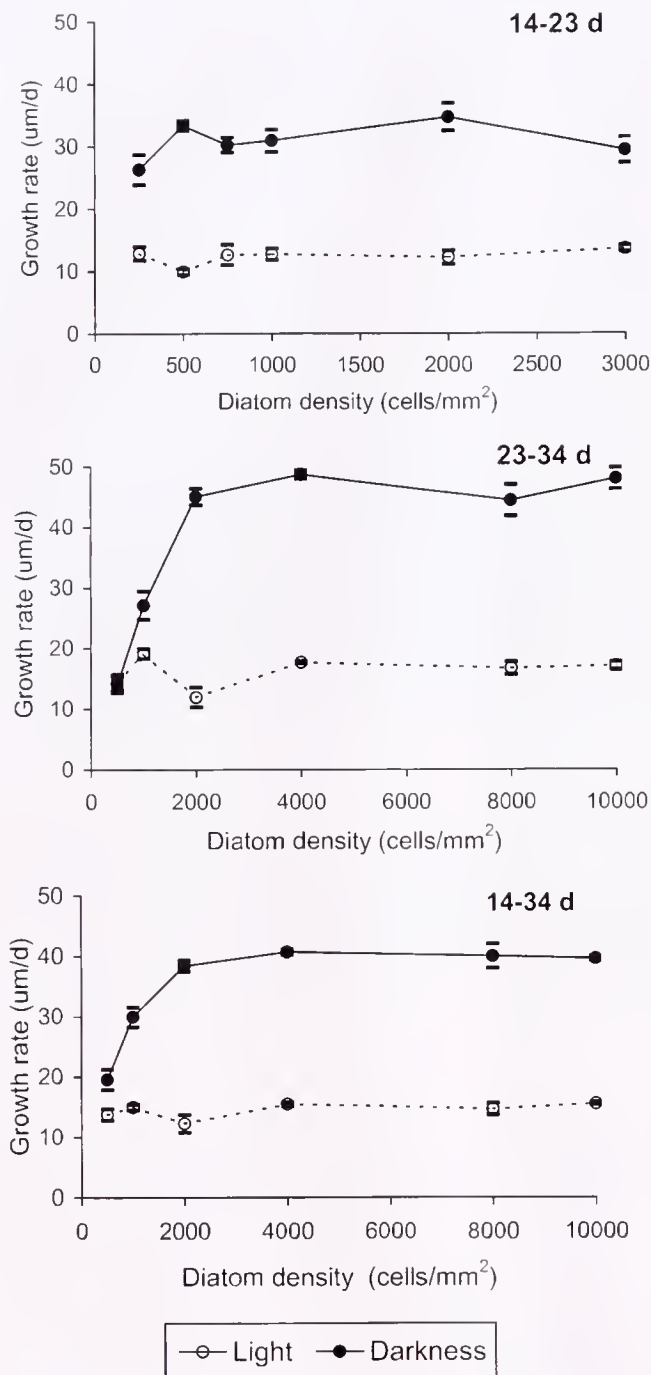


Figure 4. Growth rates (mean \pm SE) of *H. rufescens* postlarvae in Experiment 1 during different age intervals.

Effects of Diatom Density

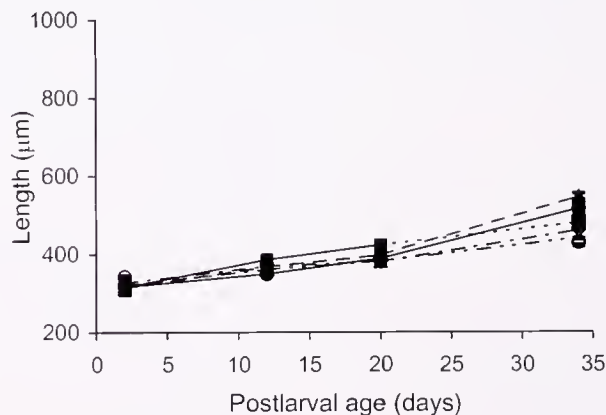
Diatoms should be available in enough quantities to produce good growth of abalone postlarvae, but very high diatom densities may have a negative impact on postlarval growth because of extreme oxygen concentrations and/or other ecologically adverse conditions (Ebert & Houk 1984, Searcy-Bernal 1996). Therefore, higher survival would be expected at intermediate diatom densities as observed in Experiment 2 under illumination (Fig. 2). However, high diatom densities under darkness did not have a negative impact on postlarval survival as discussed earlier (Fig. 2). High sur-

vival (>60%) has been reported for postlarvae of *H. discus hannai* and *H. iris* fed different diatom species at densities between 1,000 and 4,000 cells/mm² (Kawamura & Takami 1995, Kawamura et al. 1998a).

Although the effect of diatom density on initial postlarval growth was not significant in Experiment 1 (Fig. 4, Table 2) where the initial postlarval age was 14 days; a significant effect was found in Experiment 2 (Fig. 6, Table 3), where the initial postlarval age was 2 days. Growth of these newly settled postlarvae increased most markedly as diatom density increased up to 1,000 cells/mm², and this effect was stronger under darkness. Probably this reflects the benefit of increased diatom mucus and associated bacteria in the biofilms as diatom density increases, since early postlarval growth does not depend on the direct digestion of diatom cells (Kawamura et al. 1998b).

The growth of older postlarvae responded to higher diatom densities following similar patterns in both trials. The second growth period evaluated in Experiment 1 (Fig. 4) started with 23-day-old postlarvae and is comparable to the third evaluation period (Fig. 6) in Experiment 2 that started with 20-day-old postlarvae. In both cases growth rates in darkness increased sharply as

Exp. 2: Light



Exp. 2: Darkness

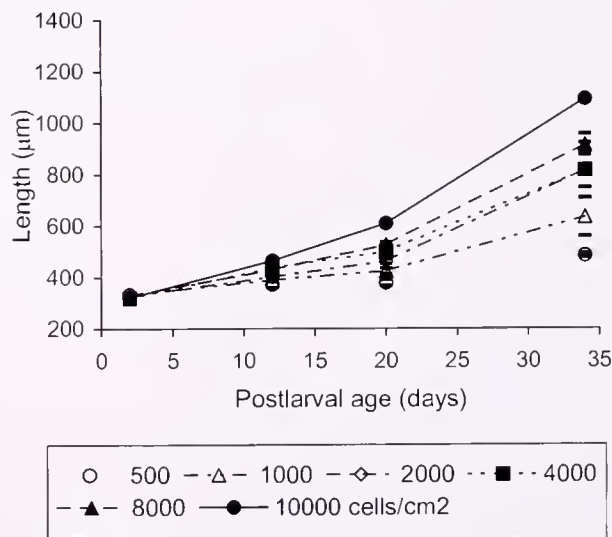


Figure 5. Shell length (mean \pm SE) of *H. rufescens* postlarvae at different ages in the experimental light conditions and densities of *N. incerta* in Experiment 2.

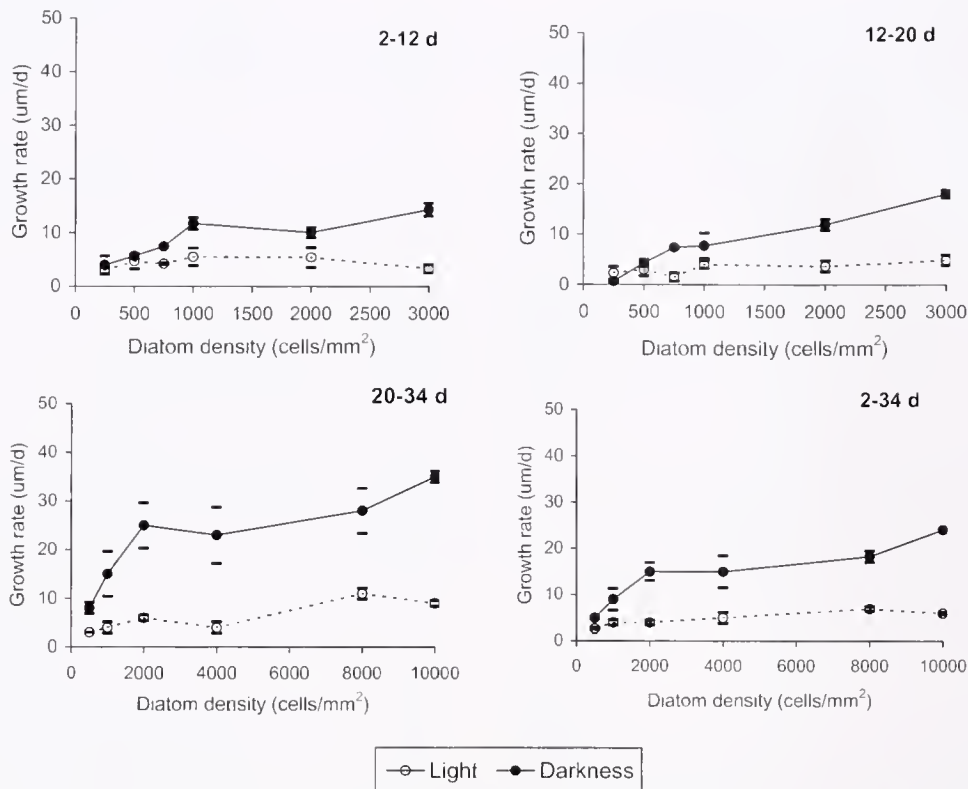


Figure 6. Growth rates (mean \pm SE) of *H. rufescens* postlarvae in Experiment 2 during different age intervals.

diatom density increased up to 2,000 cells/mm² and leveled off at higher densities. This suggests a possible optimum diatom density to feed *H. rufescens* postlarvae of these ages under darkness.

Under continuous illumination very little effect of diatom density on postlarval growth was observed in both experiments. This contrasts with the results of Searcy-Bernal et al. (2001) who showed that 15- and 30-day-old postlarvae of *H. fulgens* kept at between 17°C to 19°C and $\sim 50 \mu\text{E}/\text{m}^2/\text{s}$, increase their growth as *N. incerta* density increases up to 2,000 cells/mm². This difference might be due to several factors including experimental conditions, postlarval, or interspecific differences.

Survival and postlarval growth in Experiment 2 were lower than in Experiment 1, probably because of differences between larval batches or experimental conditions. Variability in abalone growth is common even within the same batch (Hahn 1989) and differences in postlarval growth of *H. rufescens* between batches similar to those observed here have been reported in other studies (Martínez-Ponce & Searcy-Bernal 1998). On the other hand Experiment 1 started with older postlarvae cultured under optimal conditions before the trial was conducted.

The main conclusion of this work is that the growth of abalone postlarvae might be dramatically improved if they are cultured in darkness. Large-scale trials under hatchery conditions are required to determine if this result can be extrapolated to production. The potential implications of these findings to postlarval culture would depend on a reliable source of cultured diatoms to reinoculate tanks as required, which is not currently a technical problem (Roberts et al. 2000).

ACKNOWLEDGMENTS

The authors thank the commercial farm Abulones Cultivados (Eréndira, B.C., México) for the donation of abalone larvae, Enrique Valenzuela (I.I.O.) for providing the monocultures of *N. incerta*, Casandra Anguiano-Beltrán for her technical support, Carmen Paniagua-Chávez, and two anonymous reviewers for their critical comments on the manuscript. This study was partially funded by the University of Baja California (grants 4040 and 4403) and the Mexican Government (CONACYT grant 37461-B and SNI grant 5532). This paper is part of the doctoral dissertation of E. Gorrostieta-Hurtado supported by a CONACYT scholarship.

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EFFECTS OF DENSITY AND FOOD SUPPLY ON POSTLARVAL ABALONE: BEHAVIOUR, GROWTH AND MORTALITY

ROB DAY,* PATRICK GILMOUR, AND SYLVAIN HUCHETTE

Zoology Department, The University of Melbourne, Parkville, Victoria 3010, Australia

ABSTRACT Understanding density dependent processes in abalone is important for sustainable management of fisheries, and optimum aquaculture production. Density and food were manipulated to test their interacting effects on the growth and survival of hatchery produced postlarval *Haliotis rubra*. Feeding and dispersive behavior was also examined. Initial densities ranged between the densities used in aquaculture and those reported in the natural environment. Higher initial density increased the mortality rates of postlarvae in two experiments. Simultaneous manipulation of diatom food supply and density showed that the effect of density on mortality was mostly independent of food. Growth was strongly related to food supply, but also significantly reduced by high initial density, and these effects were additive. When diatom density was low, postlarvae did not increase rasping rates so the effect was that food was consumed more slowly. This explains the strong effect of food supply on growth. No aggressive interactions between feeding postlarvae were observed, but they graze intensively, leaving small distinct patches that have been cleared of algal food. Postlarvae encountering patches without food move rapidly and apparently randomly in search of food. The dispersal rate of postlarvae increased with density, presumably because post larvae spent more time searching for food. This searching is likely to be energetically expensive, and would increase at high densities because more cleared patches are created. This may explain why high initial densities of postlarvae had persistent reduced growth even when food was abundant, but does not explain the increased mortality at high density irrespective of food supply. We hypothesize that this effect may be attributable to other factors, such as the increased transmission of a pathogen at high density.

KEY WORDS: recruitment variability, intraspecific competition, density dependence, diatoms, *Haliotis rubra*

INTRODUCTION

The role of density dependent processes in populations has been an important debate in ecology for decades (Nicholson 1933, Andrewartha & Birch 1970, Stubbs 1977, Rose et al. 2001). Understanding any density dependent processes throughout the life cycle is also vital to sustainable management of exploited populations (Fogarty et al. 1991, McShane & Naylor 1995), because the overall compensatory density dependence in the relation between adult population size and future recruitment is crucial for any sustainable fishery (McGarvey 1996, Rose et al. 2001). Density dependent processes will also determine the optimum production strategies in aquaculture.

Although it is understood that some form of density dependence must operate in all populations (Haldane 1953), recruitment to some fish and invertebrate populations is so highly variable that some have questioned whether any relation exists between adult stocks and recruitment (e.g., McShane 1995, McShane & Reyn 1996). There is general agreement that the early life history stages of these organisms are a likely source of much of the variation in recruitment, due to their small size and relatively large numbers (Hunt & Scheibling 1997). This may make them particularly vulnerable to density dependent processes such as predation (Hurlbut 1991, Shepherd & Daume 1996), competition for food (Marshall & Keough 1994, Schmitt 1996, Elmgren et al. 2001), or space (Paine 1984).

Both pre- and postsettlement stages may contribute to recruitment variability, but the early postsettlement period has not received much attention, due to the difficulty of measuring small settlers whose distribution varies greatly in time and space (Keough & Downes 1982). Measuring recruitment at some arbitrary minimum size may ignore mortality that could have occurred in the period between settlement and observation (Keough & Downes 1982, Connell 1985). In cases where settlement rates are

high (Gaines & Roughgarden 1985, Hurlbut 1991, McShane 1991) or mortality rates vary over time (Roegner 1991) postsettlement processes will be more important in determining the degree of recruitment variation than larval supply. Further, the significance of the postsettlement period is likely to be especially important in populations where larval dispersal is relatively local, because the dynamics will correspond to a closed rather than open population model (Roughgarden et al. 1988, Underwood & Fairweather 1989). In closed populations the postsettlement phase will both depend on and, with a time lag, impact on adult population density, because larval influx from other populations will not confuse the relationship.

In aquaculture the rate at which a farm can produce abalone of any given size will depend directly on the survival and growth rates of postsettlement individuals. Thus, understanding any density-dependent processes that affect growth and survival is essential for determining the most effective strategies to maximize yield and minimize turnover rates and costs in aquaculture ventures.

Abalone are ideal for examining density dependent mechanisms in the postsettlement phase, because they are now extensively cultured. The issue is important because effective management arising from an understanding of the processes underlying recruitment variation is vital to the sustainability of these fisheries (Shepherd & Rodda 2001), and there is evidence that many abalone species have localized larval dispersal (Prince et al. 1987, Prince et al. 1988, McShane et al. 1988), so that populations are effectively closed. Studies on several species have indicated that various density dependent processes may act in the early postsettlement stage. These include competition for space (McShane 1991), predation (Shepherd & Daume 1996, Preece et al. 1997), and competition for food (Daume et al. 2001, Heasman et al. 2001). While these studies show evidence for density-dependent growth or mortality, the underlying mechanisms remain obscure.

The lack of a mechanistic understanding of early life history processes can be associated with the inherent difficulty in studying tiny individuals in the field (McShane 1992). Using a suction-

*Corresponding author. E-mail: r.day@unimelb.edu.au.

sampling device McShane (1991) sampled postlarval *Haliotis rubra* in the crustose coralline red algal habitat, where abalone larvae settle almost exclusively (Shepherd & Turner 1985, Shepherd & Daume 1996, Daume et al. 1999). Over time, the results showed a change in mortality rate that was correlated to the number of postlarvae observed soon after settlement. Shepherd & Daume (1996) obtained similar results from an *in situ* study of *Haliotis laevigata* and *Haliotis scalaris*. The limitation of these approaches is that they are observational rather than experimental so that their conclusions are limited to inferences or suggestions.

Ignorance of the underlying processes behind density dependent mortality and growth extends to the hatchery environment. Because the effects of food quality and postlarval diet on growth and survival have received much attention in the recent literature, (e.g., Kawamura et al. 1998a, Kawamura et al. 1998b, Roberts et al. 1999a, Daume et al. 2000), the role of density in this has only recently been recognized (Daume et al. 2001, Heasman et al. 2001). This is surprising considering that improved settlement rates using cues such as the green alga *Ulva lens* (Daume et al. 2000, Daume et al. 2001) allow very high densities of postlarvae to be cultured. Even this recent work on postlarval density only describes the outcomes of the interaction (in terms of the effects on growth and survival). The same problem as in field studies is evident: while competition for food is proposed, the mechanisms remain untested.

The objective of this study is to identify the mechanisms behind any density dependent effects on growth and survival rates of postsettlement abalone. Identifying these mechanisms might both allow abalone farms to increase their efficiency, and prove useful in predicting the dynamics of natural populations. Small-scale experiments in a hatchery environment and observations of postlarval behavior in relation to food were used to test for intraspecific competition for food in postlarval *H. rubra*, and to investigate the underlying mechanism of any density effects. The postlarval phase in abalone has been defined as the period after settlement and metamorphosis (~300 μm shell length) to the formation of the first respiratory pore (e.g., Leighton 1974, Kawamura et al. 1998b). This seems arbitrary in an ecologic context. In this study the end of the postlarval phase is considered to be the shift to the cryptic juvenile phase at between 5- and 8-mm shell length (Shepherd & Turner 1985, Shepherd & Daume 1996), which involves a change in habitat and behavior that will influence the resources they use and sources of mortality. In the hatchery environment postlarvae are weaned off microalgal food and onto artificial food at a similar size (Hahn 1989).

METHODS

Spawning was induced in conditioned brood stock at the hatchery of Ocean Wave Seafoods, Lara, Victoria. Larvae were kept in an aerated, 300 L conical tank with flowing UV-sterilized, 3- μm filtered water. Larvae were used in experiments 5–6 days after fertilization, when they were judged competent to settle, as they began exploring the tank's surface and had developed the third tubule of the cephalic tentacle (Daume et al. 2000). The experiments used two larval batches, each the result of a mix of four to six parents.

A culture of the diatom *Navicula* sp., obtained from the Department of Fisheries, Western Australia, was used as food in all experiments. *Navicula* sp. has been shown by Daume et al. (2000) to produce high growth and survival rates in postlarval *H. rubra*.

The non-axenic culture was maintained in *f/2* growth medium complemented with silicates (Guillard & Ryther 1962) at ambient temperature and under a 12 h L:D photo cycle. For the *f/2* growth medium, seawater was filtered to 1 μm before adding sodium hypochlorite (commercial chlorine, 12.5% W/V) and aerating for 3 h. A neutralizing solution of sodium thiosulfate was then added, followed by a further 3 h of aeration.

Behavior

Ten Petri dishes (85 mm diameter) were inoculated with *Navicula* sp. and cultured for 3 days, after which diatom density was measured by counting the number of diatoms within a 300- \times 300 μm quadrat at $\times 100$ magnification for 10 random fields in each dish. Mean density was 517 mm^{-2} (SE = 24, n = 10). Isolated square patches of food approximately 5 \times 5 mm were formed by removing a 5-mm wide grid of diatoms with a small cloth. A single postlarva (mean size 1273 μm , SD 200 μm , n = 12) was placed in the center of one of these patches in each dish, and its position recorded every 4 h for a 24 h period (under a 12 h light to dark cycle).

Effect of Density on Dispersal

Plates with a cover of the green alga *Ulva lens* of at least 95% were used to examine the grazing behavior of postlarvae. A good cover of *U. lens* greatly enhances the settlement rate of *H. rubra* larvae and provides food to larger postlarvae (Daume et al. 2000, Daume et al. 2001, Daume et al. 2004). Squares (200 \times 200 mm) cut from commercial settlement plates (30 \times 60 cm) were placed horizontally in a 225 \times 225 \times 145 mm container filled with 3- μm filtered seawater with an average output of 80 \pm 5 $\text{mL}\cdot\text{min}^{-1}$ per container. Overhead fluorescent lights provided illumination on a 12 h cycle and temperature remained at 18 $^{\circ}\text{C} \pm 1$ $^{\circ}\text{C}$.

At settlement, the larvae were restrained to the center of the plates with a vertical PVC pipe, 45 mm in diameter and 16 cm high. After settlement, the pipe was removed and the larvae were allowed to spread. A grid with concentric squares (Fig. 1) was placed behind the settlement plates and the number of postlarvae within the central square was manipulated by haphazardly removing some of the postlarvae with a soft cloth to obtain high (~100) and low (~20) densities. All post larvae outside this central square were removed with a soft cloth. Five replicates were used for each treatment. The distribution of the postlarvae was then recorded by counting the individuals in each marked square of the grid at 1.5, 6, 12, 24, 48, 96, and 192 h after the removal of the pipe.

Density-light Interaction

Two levels of initial postlarval density (20,000 and 5,000 m^{-2}) were tested and the abundance of diatom food was controlled using 2 levels of illumination (one layer of shade cloth and no shading). Higher illumination maintains a higher diatom productivity and abundance (Kinne 1973), so that light management is a common method in commercial hatcheries to control algal abundance on settlement plates (Daume et al. 2001). The higher initial density was chosen according to the highest densities found in the natural environment (McShane 1991, McShane & Smith 1991, Nash et al. 1995) and the scale of the experimental units. Whereas 20,000 individuals m^{-2} is c.a. 1.6 times greater than the maximum recorded in the wild (McShane & Smith 1991) their sampling technique examined density on the scale of m^2 and small, denser aggregations may occur on a smaller scale—such as that used here.

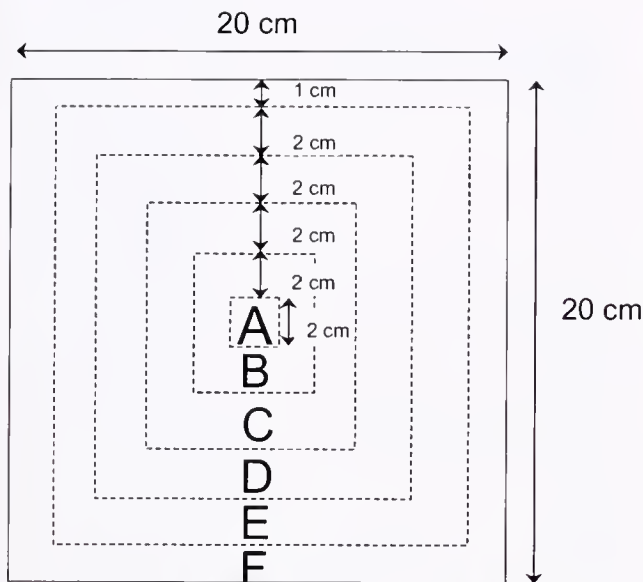


Figure 1. Grid used to assess the distribution of postlarvae during the dispersal experiment on *U. lens* covered plates. At the start of the experiment, postlarvae were restrained to the central square (A). Thereafter, counts were carried out in each area until 192 h after the postlarvae were allowed to disperse across the plate.

The lower initial density corresponds to that used in the aquaculture industry (Heasman et al. 2001), and was judged sufficient to ensure that a minimum number of postlarvae survived in each jar after settlement and initial mortality, two processes that are notoriously variable.

Commercial plastic settlement plates (30 × 60 cm) were cut into squares (5 × 5 cm). These plates had a cover of the green alga *Ulva lens* of at least 95%. Plates were then randomly assigned to glass jars (c.a. 250 mL volume) filled with 3 μ m filtered seawater. The jars were placed 35 cm below a fluorescent neon light on a 12 h L:D cycle. Approximately 300 larvae were added to each jar. There was no flow at this stage. Three days after the larvae were released the plates were examined under a dissecting microscope. The initial treatment densities were established by haphazardly removing postlarvae from the plate until the desired number had been reached. After this manipulation, numbers were recounted to ensure accuracy. A flow-through system of 3 μ m filtered seawater was turned on at this point.

Opaque PVC piping was placed around all jars to restrict illumination to that coming from above. In the shaded treatments, 70% shade cloth was fastened over the top of the PVC piping. Six replicates were used for each of the four treatment combinations. Survival and growth were monitored weekly for a period of 6 wk. For sampling, platelets were carefully transferred to a petri dish under the dissecting microscope and were kept immersed in water at all times. The postlarvae were counted, and seven randomly selected postlarvae were measured at ×50 magnification with a graticule (±20 μ m resolution). The jars were wiped clean after each weekly measurement to minimize disturbance. Average flow rate was also recorded for each outlet of the system. Because the counts were obtained repeatedly from plates over time, differences in overall mortality of post larvae between initial density treatments were tested using linear comparisons of means in a repeated measures ANOVA. We chose this method rather than survival

analysis because it is more familiar to most ecologists and the interpretation is straightforward.

Food quantity and quality

Food abundance (high and low) and initial postlarval density (20,000 and 3,500 m⁻²) were manipulated to test their combined effects on growth and survival. In the density-light experiment, food quantity and quality could not be closely monitored because diatoms could not be clearly seen through the dense *U. lens* cover. This second experiment was designed to directly manipulate and monitor the food levels and was thus carried out in petri dishes inoculated with a diatom film. The petri dishes were randomly placed under a fluorescent light (12 h L:D cycle), and water was exchanged with sterile, 3 μ m filtered water every 2 days. The experiment was run for 3 wk.

Small petri dishes were used (20, each 3.4 cm diameter, 9.1 cm² area) and a diatom film of *Navicula* sp. was grown on them before the start of the experiment. Diatom abundance was controlled in two ways. Half of the petri dishes were inoculated 4 days prior to the others to allow for a denser initial diatom biofilm. This difference was then maintained by the addition of extra diatoms to the dishes (1–2 times per week). Five replicates were used for each of the four treatment combinations.

Post-larvae were grown at low density on large (20 × 20 cm) *U. lens* plates in a 3 μ m flow-through system and then transferred 16 days after settlement to the petri dishes at a mean size of 663 μ m ($n = 118$, SD = 70 μ m). Transferral involved brushing them off the plates into a clean petri dish with a soft cloth. They were left for 3–4 h and rinsed several times with 3- μ m filtered, sterilized seawater to remove diatoms, fecal material, and the like. They were then delicately moved into the treatment dishes with a soft cloth. Damaged shells were removed and postlarvae that did not land the right way up were inverted with a small jet of water from a pipette.

Survival was checked 1, 3, and 7 days after transferral to the petri dishes and every week thereafter. The initial size of all post-larvae in the low-density treatments and 8 randomly selected post-larvae from the high initial density treatments were measured on the second day at ×50 magnification. Diatom abundance was measured at the same time with a compound microscope. Ten fields of view at ×100 magnification were randomly selected and the number of diatoms within a 500 × 500 μ m grid was counted along with an estimate of percent coverage. Growth, survival, and diatom abundances were measured weekly.

RESULTS

Behavior and Patchy Food

Postlarvae generally began grazing less than 20 sec after being transferred to a patch of food. Rasping of the surface with the radula could be observed with an inverted compound microscope and diatoms could be seen being removed by the postlarvae.

Grazing behavior of postlarvae consisted of steady rasps of the surface. After each rasp the postlarvae turned a small angle in one direction. This direction was the same until the abalone had turned approximately 120°, as shown in Figure 2. A small movement forward was made to maintain its position on the edge of the food before grazing continued in the opposite direction. Postlarvae grazed areas thoroughly and in a manner that formed bare patches of space.

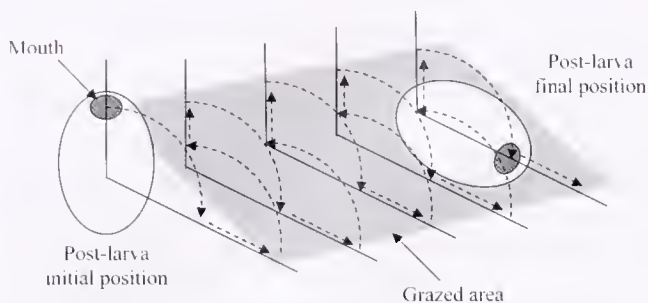


Figure 2. Movement patterns of postlarvae grazing on densely covered diatom surface.

The mean grazing rate of postlarvae was 1177 ± 113 diatoms hour^{-1} in patches of diatoms ($n = 10$). There was no significant linear regression of size on grazing rate ($F_{1,8} = 1.869$, $P = 0.209$). Observations showed that less food is consumed per unit time when diatom densities were low. Where diatoms were closer together, each rasp of the radula picked up more diatoms than when the diatoms were more widely spaced. When diatom density was minimal (approximately 50 mm^{-2}) the radula would only pick up diatoms every three to four rasps, as opposed to every one to two rasps at higher diatom densities. There was no obvious increase in rasping rate of the radula at lower diatom densities, which suggests that postlarvae do not compensate for a lower diatom density by grazing faster.

Post-larvae would often graze to the edge of patches of food. In doing so, their anterior sensory appendages lost contact with the food source. In the absence of this contact postlarvae would stop grazing and start a series of apparently random turns, often more than the 120° rotations observed when grazing. Their speed of movement also increased. This pattern of movement ceased when the postlarvae found another patch of food. Three postlarvae were observed from the time they stopped grazing to the time they found a patch and started grazing again. This occurred in a mean time of 105 ± 34 sec. Seven postlarvae were observed to leave their patch over the 28 h of the experiment. This would commonly occur before the entire patch had been consumed (mean cover remaining = $43 \pm 4\%$, $n = 7$). This does not include the postlarvae that may have traveled between patches between observation intervals. Contacts between postlarvae were observed in the other experiments, but there was no aggressive interaction: searching or grazing often continued throughout the contact.

Dispersal

Figure 3 shows the distribution of postlarvae in each concentric region expanding from the center of the plate. Postlarvae started spreading immediately and a change in distribution was observed within $1\frac{1}{2}$ h. The movement of postlarvae out of the central zone appeared faster at higher initial densities. Towards the end of the experiment, when significant numbers of postlarvae were reaching the larger outer areas of the plate, the proportions became more even across the plate at high rather than at low initial density (Fig. 4), although the proportion of postlarvae remained highest in the central area.

The Effect of Shading and Density

Figure 5 shows the change in size and the trend in log numbers surviving for the postlarvae in each of the four treatments over

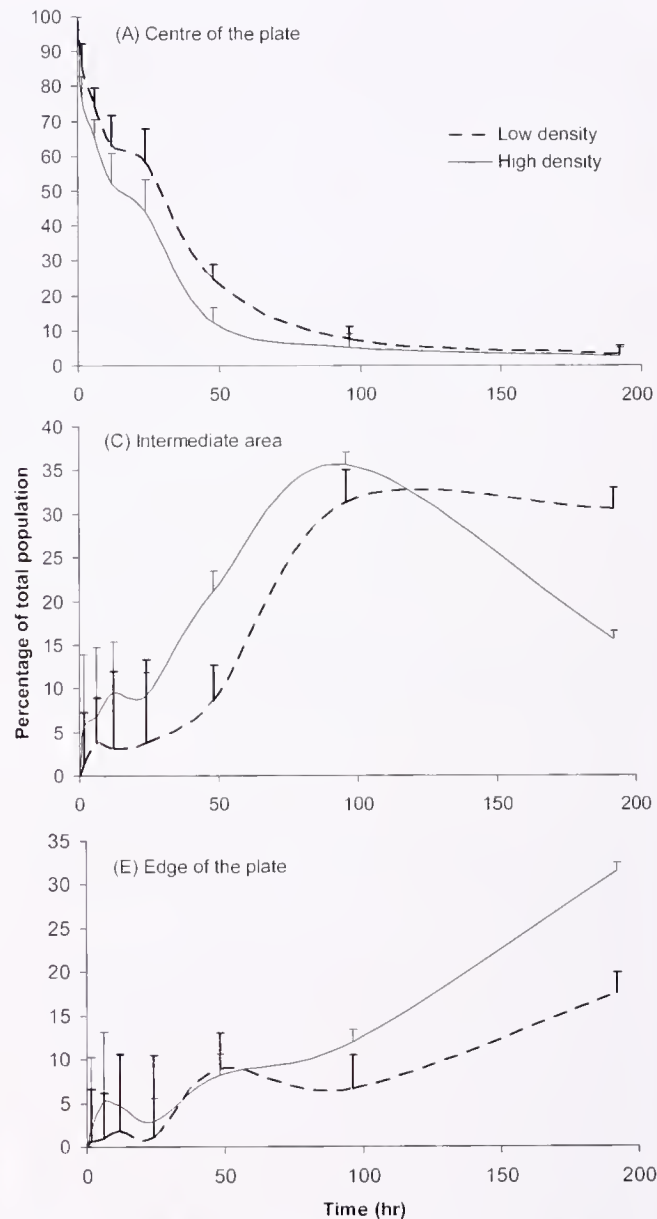


Figure 3. Dispersal of postlarvae over time on square plates covered with *U. lens*. Proportions of the population in 3 distinct areas (A, C, & E) of the plates are presented.

time. Although variation in survival within treatments was high, there was a significant interaction between time and density (Table 1), and the linear component of this interaction was significant ($F_{1,20} = 8.498$, $P = 0.009$). Thus overall mortality was significantly affected by the density treatment. There was no significant time by light interaction, which indicates that mortality is independent of the shading treatment. There was no significant difference in flow between treatments (Light: $F_{1,20} = 0.692$; Density: $F_{1,20} = 0.126$; interaction $F_{1,20} = 1.203$; $P > 0.20$ in all cases).

Analyzing the size data for the whole experiment duration would have greatly reduced the replication level in the ANOVA with repeated measures, due to the mortality of all postlarvae in some of the experimental units. Therefore only the data from the first 2 wk of the experiment were analyzed this way. In weeks 0–2 postlarvae became significantly larger in the unshaded treatments

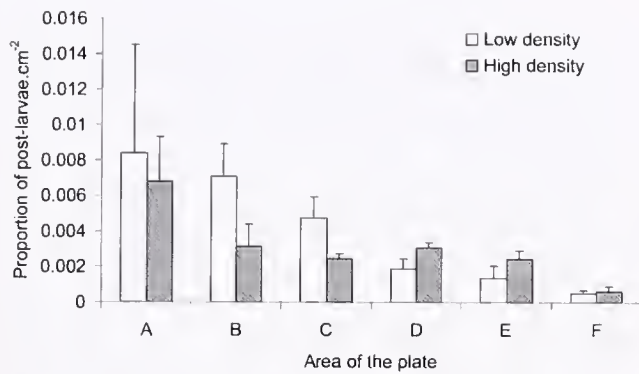


Figure 4. Density of postlarvae in each area of the plate after 192 h. A–F refer to areas of the plate shown in Figure 1.

(Table 1, Fig. 5B). There was also a trend towards smaller sizes in the high initial density treatments (Fig. 5B). This trend became significant after 5 wk (Density: $F_{1,9} = 9.80$, $P < 0.05$; Light: $F_{1,9} = 2.92$, $P = 0.12$, Interaction: $F_{1,9} = 0.407$, $P = 0.54$) despite the loss of replication.

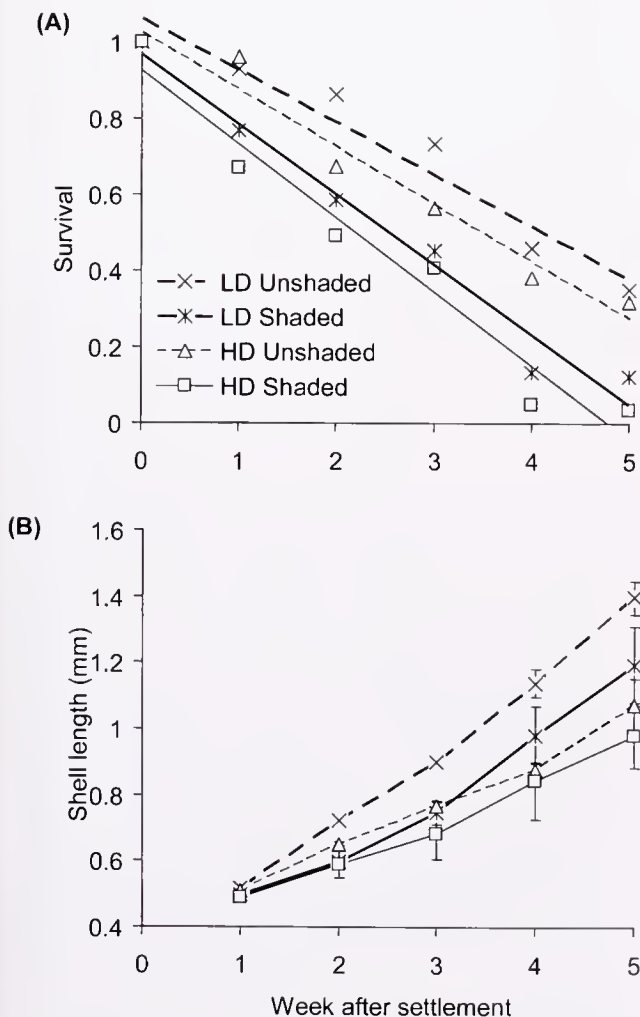


Figure 5. Survival (A) and growth (B) of postlarvae kept at 2 levels of density on shaded or unshaded plates covered with *U. lens* and diatoms.

TABLE 1.

Repeated measures ANOVA testing for the effects of density and shading on the log numbers surviving (week 0–5) and size (week 0–2) of post-larvae on plates over time. Probabilities were adjusted using the Greenhouse-Geisser statistic.

Source of Variation	Survival (Log Counts)			Mean Size		
	Df	MS	F	df	MS	F
Between plates						
Density	1	1.00	0.19	1	29172	3.79
Light	1	1.09	0.21	1	65550	8.51*
Density × Light	1	9.38	1.80	1	5602	0.73
Residual	20	5.22		13	7701	
Within plates						
Time	5	23.14	35.01*	2	285272	100.95*
Time × Density	5	2.34	3.55*	2	8022	2.84
Time × Light	5	0.88	1.34	2	12336	4.37*
Time × Density × Light	5	0.83	1.25	2	1441	0.51
Residual	100	0.66		26	2825	

* $P < 0.05$.

Food Quantity and Quality

Food abundance varied between treatments, as shown by the analysis of diatom densities (Table 2). Interestingly, while there was no significant three-way interaction with time, there was a significant interaction between density and food level. From Figure 6C, this seems to stem from the fact that diatom abundance differed more between densities at the high food level, than between densities at the low food level. Diatom abundance increased over time in the high food level treatments but was fairly constant in the low food treatments, which explains the time by food interaction. The smaller time by density effect is due mainly to the slower increase of diatom abundance in the high food treatment where density was high. Presumably this merely reflects the faster consumption of diatoms in this treatment.

The effect of food level and initial density on postlarval survival is shown in Figure 6A. For the first 14 days there was a steady decline of postlarval numbers in both high-density treatments, compared with a low and stable mortality rate in the low-density treatments. These trends seem to be independent of food availability. The repeated measures ANOVA (Table 2) reflects this pattern, as the food by time interaction and the three way interaction were not significant, whereas the time by postlarval density interaction was highly significant. From day 14–21 however, postlarval numbers also sharply decreased in the low-density treatment with excess food (Fig. 6A).

The growth of postlarvae differed significantly between density treatments and between food treatments (as shown by the time by density, and time by food tests in Table 2). Postlarvae in the low density and high food treatment were the biggest, whereas those at high initial density, but low food, were the smallest (Fig. 6B). There was no significant interaction between food and initial density (or three factor interaction with time), indicating that food level and initial density have additive effects on postlarval growth.

DISCUSSION

Behavior

There are several aspects of the observed grazing behavior of the postlarvae that seem to be important in explaining our results

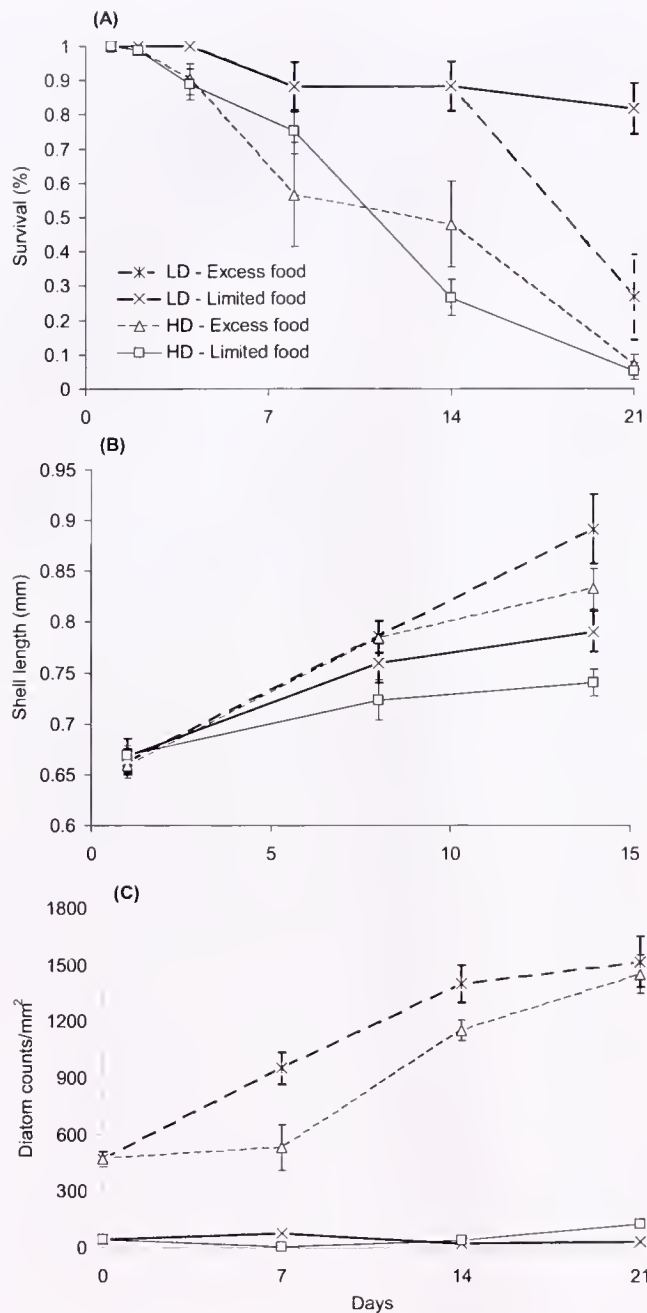


Figure 6. Effect of food quantity and postlarval density on (A) survival and (B) growth in petri dishes covered with *Navicula* sp. (C) shows the changes in density of diatom cells for the 4 different treatments over time.

and results from other experiments on abalone postlarvae by Daume et al. (2004). First, postlarvae within a patch of food were efficient grazers, removing the majority of diatoms present, so that they created small patches cleared of food. Second, food consumption in a patch appears to vary directly with diatom density. Third, postlarvae that encountered areas without food began what appeared to be a random search pattern, with rapid movements.

Rapid movement in abalone and other gastropods has been shown to be a significant energetic cost (Donovan et al. 1999). This may be due to the high levels of anaerobic respiration in-

volved, or the costs associated with mucus production for movement (Denny 1980), so that increased movement will have a significant impact on the balance between nutritional intake and energetic output (Carefoot 1987). This balance may be specially delicate in postlarvae, as compared with adults, because of the greater energetic costs associated with a large surface area to volume ratio as well as rapid morphologic development (Roberts et al. 1999a, Roberts et al. 1999b). As substantial time is spent in grazing even a small patch of algae, the time required to search for a patch may only be a small proportion of the total time spent consuming it. Nevertheless, in areas where patches of food are scarce, the sum of these erratic movements must be a significant energetic cost for the postlarvae, and impact on health and performances.

Postlarvae did not search for denser patches of diatoms. This is consistent with observations of other marine grazers and indicates an opportunistic approach to foraging (Hawkins & Hartnoll 1983). Random patterns of movement were also observed in the dispersal of postlarvae from the center of horizontal plates. If dispersal was based on the availability of food, then one might expect that postlarvae would move out in a noticeable "wave" as food became depleted in the center of the plate. The spread across the plate, with a higher proportion at the center even after 192 h, suggests instead a more random movement that was independent of food density. Dispersal was faster at higher initial densities of postlarvae. This pattern can be explained by our behavioral observations. Postlarvae at high density presumably more often encountered patches grazed by other postlarvae, which would lead to more time spent moving and thus a faster dispersal.

Similarly, postlarvae in high-density treatments would encounter many grazed patches, leading to relatively costly rapid movements. Where diatoms occur in patches of high and low density, postlarval nutrition may be even more variable, because some would spend long periods grazing areas with low food density. This food situation is likely to arise when an area has been grazed some time previously. Diatoms would have started to recolonize the grazed patches, but would not be at high density. It seems obvious that the more pregrazed areas encountered by the postlarvae, the lower the energy intake in proportion to the costs to the postlarvae.

Behavior may explain the distribution of postlarvae within the hatchery environment. Postlarvae are often grown on vertical plates covered in microalgae (Hahn 1989). Our observations of blacklip abalone on these plates a few weeks after settlement indicate a higher density on the top of the plates compared with the bottom. The top parts of the plates receive more light and are therefore likely to have denser food. Towards the bottom, grazed patches would not be quickly recolonized by diatoms, so that postlarvae may spend less time grazing in these areas. Their rapid searching would lead to dispersal from the lower parts of plates, whereas those at the top would spend more time grazing and thus move slowly. We cannot discount taxes in response to light or gravity, but in the natural environment postlarvae are found on crustose coralline algae that often have very rugose or uneven surfaces, and they occur on the tops or sides of rocks (McShane 1991, Shepherd & Turner 1985, Shepherd & Daume 1996). Thus, light and gravity may not be reliable cues to find food in natural situations.

Effect of Density on Postlarval Growth

As larvae from different batches may differ in quality (Martínez & Searcy-Bernal 1998, Daume et al. 2000), direct compari-

TABLE 2.

Repeated measures ANOVA of diatom density (day 0–21), logged post-larval counts (day 0–21) and mean size (day 0–14), testing for the effects of density and food levels. Probabilities were adjusted using the Greenhouse-Geiser statistic.

Source of Variation	Diatom Density			Survival (Log Nos)			Mean Size		
	df	MS	F	df	MS	F	df	MS	F
Between Petri Dishes									
Density	1	1239	5.4*	1	8.29	37.0*	1	9412	3.0
Food	1	144746	637.0*	1	0.01	0.1	1	29068	9.2*
Density \times Food	1	1471	6.5*	1	0.58	2.6	1	204	0.1
Residual	16	227		16	0.22		16	3166	
Within Petri Dishes									
Time	3	9243	62.4*	3	7.23	69.8*	2	115088	118.8*
Time \times Density	3	605	4.1*	3	2.71	26.2*	2	3621	3.7*
Time \times Food	3	8455	57.1*	3	0.31	3.0	2	13658	14.1*
Time \times Density \times Food	3	219	1.5	3	0.33	3.2	2	736	0.8
Residual	48	148		48	0.10		32	968	

* $P < 0.05$.

sions between batches must be examined with caution. Nevertheless, the mean size reached by postlarvae was negatively affected at high initial densities in both experiments. There are several mechanisms by which density could cause changes in postlarval size distributions. If size-dependant mortality was occurring, the death of smaller (or larger) individuals would skew the size distribution of the remaining postlarval population. This would give the impression of different growth rates between treatments. Because size-frequency distributions in the present experiments seemed normal, their small scale and the small number of individuals measured in each population prohibit definitive conclusions. Normal size distributions are usually observed in well-fed populations in the hatcheries however (Huchette 2003) suggesting that size-dependent mortality is unlikely, and the size differences reflect different growth rates.

This difference in growth rates continued to the end of the experiment despite the fact that differences in density between treatments were reduced by higher mortality in the high initial density treatments, so that densities were similar by the end of the experiment. A negative relationship between growth and density suggests there is density-dependent intraspecific competition for space or food (Stimson 1970, Branch 1975, Hughes 1986, Jarayabhand & Newkirk 1989, Parsons & Dadswell 1992, Foster & Stiven 1996). Direct competition for food has been shown for several marine gastropods (e.g., Stimson 1970). These are often limpets defending patches of algae near their home scars (Chapman & Underwood 1992). Direct competition is unlikely in abalone postlarvae due to their free-ranging foraging behavior, and no observations of aggressive confrontations were made throughout this study. The competitive interactions are therefore likely to be indirect.

Exploitative (indirect) competition for food has been shown in numerous benthic marine invertebrates, particularly herbivorous gastropods (Creese & Underwood 1982, Marshall & Keough 1994). It usually occurs when the abundance of a resource is depleted by consumers, and as a result this resource becomes limiting (Schmitt 1996). Food was a limiting factor because it significantly affected growth in both experiments. But even when food was clearly in excess in one of these experiments, the effect of density on growth appeared to have the same negative effect. It

seems that postlarval food consumption was affected by the grazing activity of conspecifics despite an abundance of food.

Grazing would have increased the patchiness of food. Even when the densities were reduced by mortality in the high initial density treatments, the food would have been very patchy due to intensive previous grazing. Postlarvae entering recently grazed bare patches would have begun costly searching movements, whereas those encountering patches with a low diatom density would have consumed food at a slower rate. In both cases their growth would be reduced, and this form of exploitative competition, which essentially reduces access to food, would increase with density, and have persistent effects.

Effect of Density on Postlarval Survival

Density dependent mortality of postlarvae was found in both experiments, and has also been found in large-scale hatchery experiments, where food limitation at high density has been suggested as the underlying cause (Heasman et al. 2001, Daume et al. 2001, Daume et al. 2004). While food limitation may cause density dependent growth, our results suggest a different process causes the density dependent mortality.

Because alternative processes such as density-dependent predation (Shepherd & Daume 1996) or competition for space (McShane 1991) are absent in the hatchery, the density dependent mortality might be due to exploitation competition leading to either starvation, or a reduction in overall health that makes the postlarvae more susceptible to other agents of mortality. Density dependent mortality of this nature has been described for several species (Creese & Underwood 1982, Marshall & Keough 1994). One would expect such mortality to be delayed depending on the energetic reserves of the postlarvae at the start of the experiments, yet mortality in the high-density treatments actually occurs soon after the start of the experiments. It seems highly unlikely that starvation would take effect so quickly, and before differences in growth start to appear. In the first 2–3 wk after settlement, growth is minimally affected by the diatom composition because of the remaining yolk reserves (Daume et al. 2000, Roberts & Lapworth 2000) and the uptake of dissolved organic matter (Manahan & Jaeckle 1992, Shilling et al. 1996), so that effects on mortality are unlikely until after this time.

Density dependent mortality would be expected to be less intense, or take longer to come into effect where the food is more abundant, because the food intake of the abalone would be substantially higher. In these experiments, mortality at high initial postlarval densities occurred at the same rate, irrespective of food level. In a large scale experiment in the hatchery (Daume et al. 2004) mortality was shown to be higher overall in the excess food treatment. This suggests that mortality is unrelated to starvation, and that some other mechanisms were at work.

Contact between individuals reduces growth in juvenile abalone, perhaps because of elevated levels of stress (Huchette et al. 2003a, Huchette et al. 2003b). At higher densities this would occur more often and might be a cause of the density-dependent mortality observed. Contacts between postlarvae however, did not seem to affect their behavior and are unlikely to cause the sharp declines in survival recorded.

Pathogens such as protists, viruses, or bacteria are well known agents of mortality in aquaculture (Bower 1987, Shepherd & Breen 1992). Although we have no direct evidence for any pathogen, higher rearing densities imply an increased contact between individuals and a higher probability that one or more individuals carry a pathogen. Increased contact between individuals or with mucus trails thus increased transmission of a pathogen at high densities, and may be the cause of the mortality that is observed here. Bower (1987) notes that smaller abalone (<1.5 mm) were far more susceptible to a pathogenic protist, so that these postlarvae may be highly vulnerable.

These density-dependent processes may simply be an additional pressure that increases the postlarvae's susceptibility to other stresses. These stresses could include aspects such as oxygen or carbon dioxide supersaturation or depletion in the diffusive boundary layer above the substratum (Searcy-Bernal 1996). This effect might account for the greater mortality that occurred after 2 wk in the excess food treatments of the second experiment. The diatoms were very abundant in these treatments by this time, so that the diurnal oxygen production, or nocturnal consumption, of the algae may have contributed to the mortality in the postlarvae. Because the contribution of the biomass of postlarvae to oxygen concentrations is not likely to be significant in comparison to diatoms, and diatoms were reduced by high larval densities, the boundary layer effect alone cannot explain the density-dependent mortality.

We tentatively conclude that the effect of density on mortality may be due to a pathogen, or a complex interaction between the factors discussed above.

Implications

When considering commercial systems these results must be interpreted with care, due to the small scale at which these experiments were conducted. Whereas small containers such as petri dishes and jars may not closely match conditions in hatcheries, their use has been widespread in abalone aquaculture research (Kawamura et al. 1998a, Roberts et al. 1999a, Roberts et al. 1999b). The fact that they can be easily manipulated makes them ideal for experiments aiming at identifying what is happening at a process level. Here, the existence of density dependent growth and mortality has been demonstrated in larger scale experiments (Heasman et al. 2001, Daume et al. 2001, Daume et al. 2004). Using small containers has allowed us to confirm that intraspecific competition for food is the cause of reduced growth at high density in postlarval *H. rubra*. Identifying that density-dependent mortality is not due to a shortage of food is also of enormous value.

From an abalone farm perspective, the efficiency of production is paramount. This often requires a balance to be found between the net biomass yield, the time taken to grow the abalone to market size and the cost of infrastructure and labor. Whereas density was shown to have a significant impact on the growth of postlarvae, food level was more important. Careful monitoring and the regular addition of supplemental diatoms would allow farms to grow postlarvae in high densities while maintaining reasonably high growth rates. The causes of density-dependent mortality have not been adequately identified, so that it is hard to say what management strategies would be most effective, but our results should encourage more work in this area. Commercial scale trials (Heasman et al. 2001, Daume et al. 2004) indicate that although survival rates are poorer, the number alive after 2 mo is still greater at high initial densities. Because production of large numbers of larvae is becoming easier, settlement of larvae at high densities may be the most efficient mode of production.

Although natural conditions are also different from those used here, it seems possible that the same density dependent mechanisms would operate on postlarvae in small patches of habitat, and these may play a role in regulating natural populations. This study highlights the importance of a process level understanding of the mechanisms underlying density-dependent growth and survival in abalone and opens up a range of new questions on these processes. Possible pathogens of postlarvae and the role of the boundary layer in the early life stages deserve further research.

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NURSERY CULTURE OF THE ABALONE *HALIOTIS LAEVIGATA*: LARVAL SETTLEMENT AND JUVENILE PRODUCTION USING CULTURED ALGAE OR FORMULATED FEED

SABINE DAUME^{1,*} AND STEPHEN RYAN^{1,2}

¹Department of Fisheries, Research Division, PO Box 20, North Beach, WA 6920, Australia;

²cf- Great Southern Marine Hatcheries, PO Box L34, Little Grove, Albany WA 6330, Australia

ABSTRACT This study was conducted to investigate the settlement, growth, survival, and size variability of the abalone *Haliotis laevigata* on commercial scale. At settlement, two plate conditioning times for the green alga *Ulva lens* (conditioned for 6 or 8 wk) were evaluated and compared with plates colonized by different diatom species (*Navicula cf. jeffreysi* and *Cocconeis* sp.). In a choice experiment an overall settlement rate of 87% was estimated 3 days after larval release. The majority of the larvae chose to settle on *U. lens* (61% on 8-wk-old and 14% on 6-wk-old *U. lens*, with average algal cover of 97% and 82% respectively). Larvae showed a clear preference for older than for younger *U. lens*, with similar percentage cover, indicating that the developmental stage of the alga and not percentage cover per se is important in settlement induction. Only 7% and 5% respectively of the settled larvae were found on the plates colonized by the diatom species *N. cf. jeffreysi* and *Cocconeis* sp. Results clearly demonstrate that *U. lens* provides a suitable substrate to improve the settlement of *Haliotis laevigata* larvae on commercial scale. Juvenile growth ($\mu\text{m day}^{-1}$) on a mixed diet of *U. lens* and *N. cf. jeffreysi* was not constant and increased with size to 70–80 $\mu\text{m day}^{-1}$. Both growth rate and size variability increased over time until juveniles reached approximately 5 mm in shell length. The survival declined to ca. 30% at 44 days after settlement but stabilized thereafter. Distinct increases in size variability were observed between 3 and 16 and between 30 and 44 days after settlement, followed by elevated growth rates and a change from monomodal to multimodal distribution at day 44. The latter coincided with a shift in diet from a diatom-dominated diet to a macroalgal diet of *U. lens* at day 44. The increase in growth rate between 3 and 16 days is believed to reflect a shift in nutrition with postlarvae switching from absorbing yolk reserves to efficient exogenous feeding. The growth, survival, and size variability of juveniles was assessed when feeding on algal diets as well as a formulated diet. Juveniles grew faster on *U. lens* than on a formulated feed or plates colonized with the diatom *N. cf. jeffreysi*. Results indicate that growth of juveniles may be more variable when feeding on natural feed (*Navicula cf. jeffreysi*, *U. lens*) than on a formulated feed. However, until a feed is formulated that can match the mean growth rates achieved on *U. lens*, we suggest keeping juveniles on plates colonized with *U. lens* as long as possible. Juvenile *H. laevigata* should not be weaned onto formulated feed until they reach at least 5 mm in shell length.

KEY WORDS: abalone, algae, diatoms, growth, size variability, settlement, *Ulva lens*

INTRODUCTION

Australia has one of the world's largest abalone fisheries and its abalone aquaculture industry is expanding (Fleming 2000). With wild abalone fisheries declining in other countries, the interest in abalone aquaculture has increased substantially.

Since the inception of abalone aquaculture in Australia, research has focused on achieving the best growth rates possible in growout systems. This is because any increase in growth rates reduces production time and will usually have large cost benefits for an abalone farm. Recently it has been shown that differences in early growth of the abalone *Haliotis rubra* Leach persisted and were amplified by the end of a 4-mo growth trial (Daume et al. 2004), suggesting that early growth is important in determining later performance. Despite this result and the consequent benefits to the abalone aquaculturist, very little emphasis has been placed on improving growth rates of juvenile abalone.

Laboratory experiments have shown that the macroalgae *Ulva lens* Crouch is a suitable settlement inducer for larvae of the abalone *Haliotis rubra* Leach and *H. laevigata* Donovan (Daume et al. 2000). These findings were recently verified in the nursery for *H. rubra* (Daume et al. 2004). However, the growth rates of postlarvae (up to 3 mm in shell length) feeding on *U. lens* have been less promising and significantly better growth was achieved with benthic diatom species (Daume et al. 2000).

Formulated feeds have long been recognized to have beneficial effects for the growth and survival of juvenile abalone (Fleming et al. 1996). Experiments conducted in Australia showed that juve-

nile abalone can achieve maximum growth rates of up to 53 $\mu\text{m day}^{-1}$, and 90 $\mu\text{m day}^{-1}$ for animals ranging between 3 and 18 mm and 7 and 20 mm in shell lengths respectively when feeding on an unspecified formulated diet (Table 6 in Fleming et al. 1996). There are several studies on growth and survival of abalone juveniles when feeding on different algal species and growth rates of 60–100 $\mu\text{m day}^{-1}$ can be expected for juveniles at 3–4 mm in shell length (see review by Kawamura et al. 1998a). Some studies compared formulated diets to an algal diet. For example higher growth-rates were achieved with juvenile abalone (*H. fulgens*), of approximately 13 mm in shell length, feeding on a formulated diet compared with juveniles feeding on macroalgae (Viana et al. 1993). Corazani and Illanes (1998) showed a similar trend with larger abalone of *H. rufescens* (~21 mm in shell length). Knauer et al. (1996) found no significant difference in growth rates of *Haliotis midiae* juveniles (3–11 mm in shell length) when comparing a diatom diet (50 $\mu\text{m day}^{-1}$) and a formulated diet (59 $\mu\text{m day}^{-1}$). However the species composition and density of diatom species was not given in this study. Results of these studies indicate that formulated diets may support similar or better growth in larger juveniles, however abalone farmers still have to rely on algae as a settlement inducer and as a food source for recently settled post-larvae. Significantly better growth rates on an algal diet are expected for postlarvae and young juveniles.

Growth in abalone is highly variable (Day & Fleming 1992). The patchiness of natural food on the settlement plates is likely to contribute to the large size variation of juveniles experienced in nurseries. Formulated feed may allow a more constant form of nutrition potentially resulting in less size variation at the end of the nursery phase. By using formulated feed, farmers would become

*Corresponding author. E-mail: sdaume@fish.wa.gov.au

independent from seasonal variation in food supply and reduce variation between sites, tanks, and even plates. Formulated feed would also provide a great advantage when algal food becomes limited at the later stages of the nursery phase when juvenile grazing pressure is very high.

In this study three experiments were conducted to investigate settlement as well as growth and survival during the early development of *Haliotis laevis* when feeding on algal as well as a formulated diet. The size variability of animals in all treatments was assessed over time to determine if juvenile populations feeding on formulated feed show less size variation.

Experiment 1: A large-scale settlement experiment was conducted to test the suitability of different algal species as settlement cues for *H. laevis* larvae in a commercial abalone nursery. At settlement, diatom films dominated by the cultured diatom *Navicula cf. jeffreyi* or *Cocconeis* sp. were compared with plates covered by the green alga *U. lens* at two different developmental stages (6 and 8 wk old). **Experiment 2:** Settlement, growth and survival were estimated on a mixed diet of cultured algae (*U. lens* and *Navicula cf. jeffreyi*). Previous work has shown that nursery plates seeded with *U. lens* result in high and consistent settlement, whereas the diatom *N. cf. jeffreyi* support rapid growth of young postlarvae (Daume et al. 2000, Daume et al. 2004). This experiment was conducted to investigate if regular inoculation at commercial scale with the diatom *N. cf. jeffreyi* can sustain rapid growth until juveniles reach ca. 4 mm in shell length. **Experiment 3:** The potential for a shorter algal-reliant nursery approach was investigated by weaning young juveniles (3.79 ± 0.06 mm in shell length) onto formulated feed 10 wk after settlement. This short-term approach was compared with the traditional long-term nursery system utilizing cultured algae. The natural food was provided separately to determine which of the algal diets *U. lens* or *N. cf. jeffreyi* achieves the better growth-rates in juveniles >3 mm in shell length.

METHODS

Location

Experiments were conducted at ambient temperature at a commercial abalone farm, Great Southern Marine Hatcheries (GSMH), Albany, Western Australia between November 2001 and June 2002. The farm provided the abalone larvae and the diatom inocula for the trials.

Experiment 1: Larval Settlement on Different Algal Species

Preparation of Tanks and Plates

Three semicommercial nursery tanks (490 L) were each set up with 3 baskets in series that each held 20 settlement plates (= 60 plates/tank). Airlines were installed on the tank bottom along each side and down the center of the line of baskets (3 airlines/tank). Four treatments were tested: 6- and 8-wk-old *U. lens* and the diatom species *Cocconeis* sp. and *Navicula cf. jeffreyi*, both cultured for 2–3 wk on the plates. Five plates of each treatment were alternated so that each tank received 15 plates of each treatment, thus providing 45 plates for each treatment in total.

Algal culture

***Ulva lens* seeding:** To produce mass spore release, plates with large mature patches of *U. lens* and well-developed sporangia

were selected, wiped clean to remove any diatom film, and stored in 1- μ m filtered seawater under two layers of 70% shading cloth, 2 wk prior to starting the conditioning of the experimental plates. *U. lens* seed plates were then placed between the baskets of the experimental tanks (4 seed plates per 1000 L tank), while the tanks were maintained with no water flow, low aeration, and without shading. A complete f/2 mix, including silicates, was applied (40 g 1000 L⁻¹ Microalgae Food, Manutech, Port Lincoln, Australia). These methods for spore collection were adapted from Takahashi and Koganezawa (1988). The release of zoospores is triggered by the increase in water temperature, nutrients and light. The largest release occurred 4–5 days after the introduction of seed plates.

Diatom Culture and Inoculation

Cultures of *Navicula cf. jeffreyi* and *Cocconeis* sp. were established in horizontally laid algal bags progressively increased in size up to commercial size bags of ca. 1 \times 2 m. The diatom culture was harvested during the exponential growth phase (4–6 days after inoculation) and mixed into suspension. The diatom density was determined before tanks were inoculated. A 15 L inoculum (10^5 – 10^6 cells mL⁻¹) was used in each tank. A complete f/2 mix was applied (40 g 1000 L⁻¹). The tanks remained static with low aeration for 24 h and then received low water flow with light aeration for 2–3 days.

Diatom Cell Density and Percentage Cover of *Ulva lens*

The number of diatom cells and the percentage cover of *U. lens* were estimated under an inverted compound microscope in 15 randomly chosen fields of view on three sub samples (2 \times 2 cm) of six plates per treatment at the time of settlement.

Larval Settlement

One hundred thousand larvae were released into each tank and left with very low water flow and low aeration for 3 days. Banjo sieves (118 μ m) were fitted onto each standpipe until settlement to prevent the escape of larvae. The settlement rates of larvae were estimated on six whole plates (30 \times 40 cm) per tank after 3 days. Plates were kept submerged in seawater during counts and replaced into tanks immediately after measurements. A grid with equal size squares was placed inside the tray and a stereomicroscope was mounted above the tray to count and assess the settled larvae.

Experiment 2: Settlement and Growth on a Combined Algal Diet (*Ulva lens* and *Navicula cf. jeffreyi*)

Preparation of Tanks and Plates

The same tank set up was used as in the previous experiment. Three tanks with 60 plates each were conditioned with *U. lens* 7 days before larval settlement and then inoculated with 15 L of cultured *N. cf. jeffreyi* per tank, 4 days before settlement. The seawater was filtered to 1 μ m and provided with a flow rate of 5–7 L min⁻¹.

Larval Settlement

One hundred thousand larvae were released into each tank and left with very low water flow and low aeration for 3 days. The settlement rates of larvae were estimated on whole plates after 3 days (see earlier).

Algal Inoculation

Tanks were inoculated with 15 L of *Navicula cf. jeffreysi* culture, 4 days before and 30 days after larval settlement and every 2 wk thereafter until the end of the trial. The details of algal culture and inoculation are described earlier.

Diatom Cell Density and Percentage Cover of *U. lens*

The number of diatom cells and the percentage cover of *U. lens* were estimated in 12 randomly chosen fields of view of six plates per tank under an inverted compound microscope at $\times 200$ magnification.

Growth and Survival of Juveniles

Juveniles were reared on a combined diet of *U. lens* and diatoms until they reached approximately 3 mm in shell length. Counts and measurements of the abalone were undertaken under a dissection microscope on six plates per tank at approximately 2-wk intervals.

Experiment 3: Assessment of Different Nursery Approaches

Early Weaning Onto Formulated Feed Versus Algal Feed

After 10 wk, when juveniles reached an average of 3 mm in shell length in the preceding experiment, all animals were taken off the plates and randomly assigned to 3 different treatments. (1) A subsample of 7,500 animals were transferred into three experimental raceways (1 m \times 300 mm \times 30 mm) and stocked at a density of 2,500 animals each (70% cover). Abalone were weighed and gradually introduced to a commercial weaner diet over the next 2–3 days. Animals were fed to excess at 1% of their body weight every day for the duration of the experiment. (2) Three semicommercial nursery tanks (see previous experiments) were set up with plates covered by *Ulvella lens*. (3) Three additional nursery tanks were stocked with plates colonized by *Navicula cf. jeffreysi*. The abalone were placed onto one horizontal settlement plate on top of each basket and left for 24 h allowing them to redistribute across all feed plates. Animals reached a stocking density of ca. 50 animals per plates (ca. 3,000 per tank). All tanks and raceways had separate inlets and outlets. The seawater was filtered to 5 μ m and provided at a flow rate of 5–7 L per min⁻¹.

Algal Inoculation

Tanks were inoculated with 15 L of *Navicula cf. jeffreysi* culture, 2 wk before the experiment started and every 2 wk thereafter. The details of algal culture and inoculation are described above.

Diatom Cell Density and Percentage Cover of *Ulvella Lens*

The number of diatom cells and the percentage cover of *U. lens* were estimated in 12 randomly chosen fields of view of six plates per tank under an inverted compound microscope at $\times 200$ magnification.

Proximate Composition of Juvenile Diets

Diatom samples (*Navicula cf. jeffreysi*) were harvested and washed onto preweight 47 mm fiberglass filter paper using 0.5 M ammonium formate to remove residual salts. Samples of *Ulvella lens* were scraped of the settlement plates and washed with ammonium formate. All samples were then freeze-dried, reweighed and, together with samples of the formulated feed, forwarded to the State Chemistry Laboratory, Werribee, Victoria, for a proximate analysis of protein, carbohydrate, and fat content.

Growth and Survival of Juveniles

Growth-rates and survival of abalone were determined by measuring the size of 72 juveniles per tank and raceway as well as counting the number of juveniles per tank and raceway every 2 wk. At 10 wk, when the algal food supply became limiting, animals were taken off the old plates and moved onto a new set of plates with the same algal diet. The experiment was terminated after an additional 6 wk when animals reached 10 mm in shell length in one of the treatments.

Data Analysis

Statistical analyses were carried out using the STATISTICA computer packages. The assumptions of normality and homogeneity of variance of residuals and normal quartile plots were checked graphically for each data set using boxplots.

Daume et al. (1999) showed that the attractiveness of the substratum changes with the presence of recently settled conspecifics. Consequently, individual settlement choices are not independent, so that the numbers of larvae on the plates were analyzed using a paired *t*-test. Larvae started settling earlier on *U. lens* (old *Ulvella*-OU, young *Ulvella*-YU) than on the two diatom species (*Navicula cf. jeffreysi*, -N, *Cocconeis* sp., -C). Paired *t*-tests were performed on (1) the difference between OU and YU within each tank, (2) the difference between the average of the *Ulvella* treatments (OU + YU/2) and N, and (3) the difference between the average of the *Ulvella* treatments (OU + YU/2) and C. Total settlement rates per tank were analyzed using a 2-way ANOVA with tank and basket position as factors.

Abalone shell length measurements in the early weaning experiment were analyzed by repeated measure analyses of variance. Data at the end of the experiment was also analyzed using a 1-way ANOVA with Tukey HSD test. Relationships between the growth rates of the juveniles and the % cover of the feed species were explored with a simple regression analysis. Size-frequency distributions were drawn and compared over time and between treatments where appropriate using descriptive statistics (size range, mean, standard deviation [SD], and coefficient of variation [CV]). The shape of the distributions was compared with a Normal distribution using Shapiro-Wilks W tests (STATISTICA). Inter quartile differences IQD between the first and third quartile were used as a measure of spread of the size-frequency distributions.

RESULTS

Experiment 1: Larval Settlement on Different Algal Species

Larval Settlement

An overall settlement rate of 87% was estimated 3 days after larval release (Table 1). Sixty-one percent of the larvae chose to settle on the old *U. lens*, 14% on the young *U. lens*, 7% on the diatom species *Navicula cf. jeffreysi* and 5% on the diatom *Cocconeis* sp. We detected a high variation between tanks (ANOVA, *P*

TABLE 1.
Percentage settlement (\pm SE) of *Haliotis laevigata* after 3 days (*n* = 3).

Treatments	Old <i>U. lens</i>	Young <i>U. lens</i>	<i>Navicula</i> sp.	<i>Cocconeis</i> sp.	Σ
% Settlement	61 \pm 14	14 \pm 1	7 \pm 0.3	5 \pm 0.5	87

= 0.025) and within tanks (ANOVA, $P < 0.001$). Plates closest to the outlet had more settled larvae than plates closest to the inlet. Old and new *U. lens* were significantly different (paired $t = 4.64$, $df = 2$, $P = 0.043$). In addition, there was a significant difference between the combined *U. lens* treatments (OU + YU/2) and both diatom treatments, *N. cf. jeffreyi* ($t = 4.412$, $df = 2$, $P = 0.048$) and *Cocconeis* sp. ($t = 4.804$, $df = 2$, $P = 0.041$).

At the time of settlement, the estimated percentage cover of the old *U. lens* was 97% and 82% on young *U. lens*. A cell density of 4,978 cells cm^{-2} was estimated on plates in the *Cocconeis* sp. treatment and 150,000 cells cm^{-2} on plates with *N. cf. jeffreyi*.

Experiment 2: Settlement and Growth on a Combined Algal Diet (*Ulvella lens* and *Navicula cf. jeffreyi*)

Larval Settlement

A settlement rate of $30\% \pm 6.5$ was estimated 3 days after larvae release. There was no significant difference between tanks (ANOVA, $P = 0.47$) or within tanks ($P = 0.36$).

Ulvella Lens Cover and Diatom Density

Ulvella lens covered between 50% and 70% of the plates for most of the growing period but around 58 days after larval release the cover decreased substantially (Fig. 1). Tanks were first inoculated with the cultured diatom *Navicula cf. jeffreyi* 4 days before larval settlement. Inoculations were repeated 30 days after settlement and then every 14 days thereafter.

At the time of settlement *U. lens* covered approximately 55% of the plate area (Fig. 1). *Nitzschia* sp. was the dominant species in the diatom assemblage on the plates with an estimate of 10,489 cells cm^{-2} . This species developed naturally on the plates. The cultured diatom *N. cf. jeffreyi* slowly increased over time from 533 cells cm^{-2} at the time of settlement to ca. 36,000 cells cm^{-2} and was the dominant diatom species from day 44 until the end of the experiment.

Growth, Survival and Size Variability of Juveniles <4 Mm in Shell Length

The water temperature during this trial ranged between 17 °C and 24 °C; with an average of 20 °C. Juveniles growing on a mixed

diet of cultured algae (*Ulvella lens*, *Navicula cf. jeffreyi*) reached 3.9 mm in shell length 72 days after larval release (Table 2). The growth-rates during the first 3 days after larval release were low; averages of approximately $40\text{-}\mu\text{m day}^{-1}$ were achieved during the following 41 days and ca. $69\text{-}\mu\text{m day}^{-1}$ during the last 28 days of the experiment. Post-settlement survival was estimated at 46%, 16 days after larval release, decreasing to 29% at 44 days and to 27% at the end of the experiment. The size range of juveniles increased substantially over time (Fig. 2, Table 3). After 10 wk the size of juveniles ranged between 2 and 5.1 mm in shell length and was nearly twice as large as ca. 2 wk earlier and about 37 times larger than at 3 days after larval release (Table 3). The SD, CV, and IQD increased over time. A large increase in at least one of the above measures was observed between 3 and 16 days, 30 and 44 days, and between 58 and 72 days. The skewness of the size frequency distribution changed from negative to positive between 16 and 30 days and between 44 and 58 days.

Experiment 3: Assessment of Different Nursery Approaches

Early Weaning Onto Formulated Feed Versus Algal Feed

The average water temperature declined from 19 °C during the first 8 wk to 17 °C during the second half of the experiment (Table 4). Growth rates and survival rates were highest on *U. lens*. Juveniles feeding on *U. lens* reached 10 mm in shell length in <15 wk (Table 5). Juveniles feeding on the diatom *Navicula cf. jeffreyi* or the formulated feed only reached about 5 mm in shell length at the end of the experiment, resulting in a significant difference between the treatments (ANOVA, $P < 0.001$). Animals feeding on *U. lens* were significantly larger than the ones feeding on the diatom *N. cf. jeffreyi* (Tukey posthoc test, $P < 0.001$) and on the formulated feed (Tukey posthoc test, $P < 0.001$).

The size range of juveniles from all three treatments increased substantially over time (Fig. 3, Table 5). After 16 wk the size range of juveniles from all three treatments was highest and varied between 7.6 (formulated feed) and 8.1 mm (*N. cf. jeffreyi*) to 13.9 mm (*U. lens*). The CV ranged between 0.2 and 0.3 in all treatments and was highest in the population feeding on the diatom *N. cf. jeffreyi* after 4 wk. There was a significant difference in SD between the treatments throughout the experiment (ANOVA, $P = 0.049$). The SD was significantly lower among juveniles feeding on formulated feed than those feeding on *U. lens* at 4 wk (Tukey posthoc test, $P = 0.01$), 6 wk ($P = 0.02$), and 8 wk ($P = 0.002$) and higher among juveniles feeding on *U. lens* than in populations of the other two treatments at 10 wk (Tukey posthoc, $P < 0.05$). 12

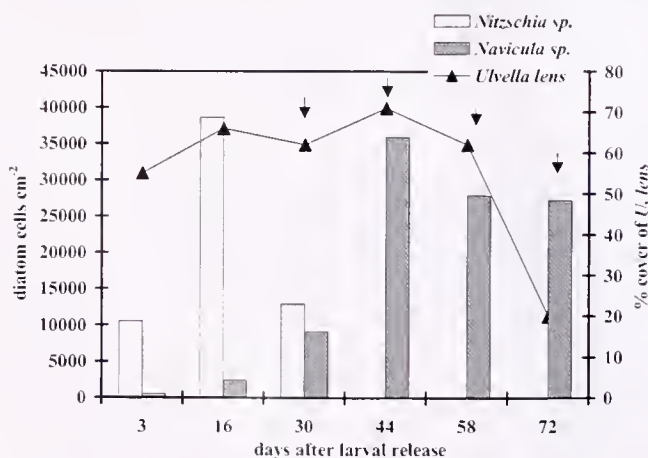


Figure 1. Cell density of diatoms and cover of *Ulvella lens* 3, 16, 30, 44, 58, and 72 days after larval release. Arrows indicate inoculation with *Navicula cf. jeffreyi*—inoculation 4 days prior to settlement not shown (Experiment 2).

TABLE 2.

Shell length (μm) and growth-rates ($\mu\text{m day}^{-1}$) and survival (%) of post-larvae as well as water average water temperature 3, 16, 30, 44, 58, and 72 days after larval release.

	3	16	30	44	58	72
Shell length	338.36	749.48	1356.48	1921.58	2890.33	3850.22
Growth-rates*	26.12	37.37	43.36	40.36	69.20	68.56
Survival†		46	37	29	28	27
Temperature*	18.37	18.75	19.43	20.42	20.45	19.62

* Between consecutive sampling times.

† Cumulative survival based on 100% post-settlement. Larval settlement at day 3 was 30%.

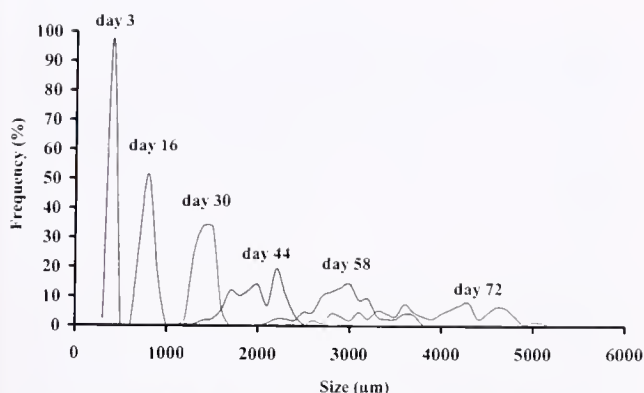


Figure 2. Changes in size-frequency distribution of *Haliotis laevigata* juveniles 1, 16, 30, 44, 58, and 72 days after larval release (Experiment 2).

wk (Tukey posthoc, $P < 0.001$), and 16 wk (Tukey posthoc, $P < 0.05$). The IQD was higher in the juvenile population feeding on *U. lens* than in the juvenile populations of the other two treatments at 12 wk (Tukey posthoc, $P < 0.001$) and 16 wk ($P < 0.05$). However, when the mean size of the populations was taken into account, the IQD/mean was highest in the *N. cf. jeffreyi* treatment.

Ulvella Lens Cover and Diatom Density

At the start of the early weaning trial, *U. lens* covered about 55% of the plate area. The cover decreased continuously until only 11% of the plates were covered after 9 wk (Table 6). The cell density on the plates in the *Navicula cf. jeffreyi* treatment followed a similar trend. New plates were introduced at 10 wk with a higher % cover in the *U. lens* treatment and higher cell densities in the *N. cf. jeffreyi* treatment than at the start of the trial. *U. lens* cover and *N. cf. jeffreyi* cell density declined rapidly.

Proximate Composition of Juvenile Diets

Protein was highest in *U. lens* and lowest in samples from the diatom *Navicula cf. jeffreyi* (Table 7). All diets contained similar amounts of fat and ranged from 4% to 7%. The formulated diet was high in carbohydrates compared with other diets.

DISCUSSION

Settlement, Growth, Survival, and Size Variability

The findings of the first experiment clearly demonstrate that the green alga *Ulvella lens* provides a suitable substrate to improve the

TABLE 3.

Descriptive statistics of juvenile size (μm) 3, 16, 30, 44, 58, and 72 days after larval release.

	3	16	30	44	58	72
Mean	338	749	1361	1926	2893	3850
Min	295	610	1158	1184	2000	2048
Max	379	863	1600	2421	3738	5100
Size range	84	253	442	1237	1690	3100
Standard Deviation	15.11	60.32	92.83	260.75	360.99	663.85
CV (%)	4	8	7	14	12	17
Inter-quartile differences	21.05	105.25	152.61	374.92	460.8	1025
Skewness	-0.34	-0.37	0.18	-0.35	0.15	0.27

TABLE 4.

Growth-rates ($\mu\text{m day}^{-1} \pm \text{SE}$) and percent survival of juveniles feeding on *Ulvella lens* or *Navicula sp.* in comparison to formulated feed as well as average water temperature.

	Week 2–8	Week 9–14	Percent survival up to 14 weeks
<i>Ulvella lens</i>	83.56 ± 8.7	62.67 ± 9.1	82.96 ± 1.4
<i>Navicula sp.</i>	20.44 ± 8.2	16.05 ± 9.6	76.16 ± 2.9
Formulated feed	13.54 ± 7.5	26.63 ± 8.8	67.06 ± 3.0
Temperature	19.14	16.67	

settlement of *Haliotis laevigata* larvae on a commercial scale. Settlement was significantly higher in both *U. lens* treatments when compared with the diatom treatments, indicating that *U. lens* is more suitable to induce settlement of *H. laevigata* larvae than a monospecific diatom film. It also indicates that larvae can distinguish between different developmental stages of *U. lens*. Similar to these findings, the settlement of *H. rubra* larvae was higher on older compared with younger *U. lens* (Daume et al. 2001). In contrast to the previous study, *U. lens* covered the settlement plates almost completely in both treatments of experiment 1 (97% and 82% for old and young *U. lens* respectively), indicating that the developmental stage of the alga (such as the development of sporangia) may be more important for the settlement induction than the percentage cover *per se*.

TABLE 5.

Descriptive statistics of size variability juvenile populations feeding on *Ulvella lens*, *Navicula sp.*, or formulated feed after 1, 4, 8, 12, and 16 weeks (Experiment 3).

	1	4	8	12	16
<i>Ulvella lens</i>					
Mean	3.91	5.24	7.78	9.20	10.13
Size range	3.70	5.00	7.30	7.70	13.90
S.D.	0.79	1.07	1.34	1.49	2.05
C.V. (%)	20	21	17	16	20
Inter-quartile differences (IQD)	1.10	1.50	1.70	2.20	2.70
IQD/mean	0.28	0.29	0.22	0.24	0.27
Skewness	-0.44	-0.26	-0.57	-0.31	-0.36
<i>Navicula sp.</i>					
Mean	3.86	3.66	4.88	5.01	5.63
Size range	3.50	4.80	5.50	6.00	8.10
S.D.	0.77	1.07	1.15	0.97	1.41
C.V. (%)	20	29	24	19	25
Inter-quartile differences (IQD)	1.00	1.80	1.63	1.20	1.63
IQD/mean	0.26	0.49	0.33	0.24	0.29
Skewness	-0.22	0.12	-0.08	0.56	0.74
Formulated feed					
Mean	3.56	3.53	4.15	5.00	6.77
Size range	3.50	3.30	3.50	6.30	7.60
S.D.	0.86	0.77	0.78	1.10	1.31
C.V. (%)	24	22	19	22	19
Inter-quartile differences (IQD)	1.23	1.13	1.10	1.20	1.63
IQD/mean	0.34	0.32	0.26	0.24	0.24
Skewness	0.15	0.02	0.13	0.70	0.25

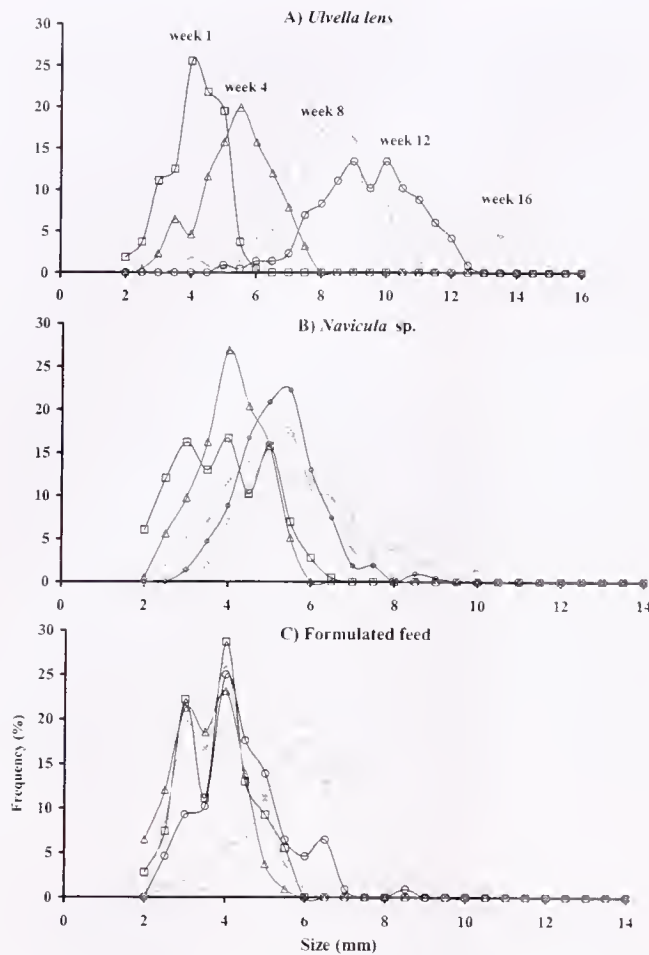


Figure 3. Size-frequency distribution of juveniles feeding on (A) *Ulvella lens*, (B) *Navicula cf. jeffreyi*, and (C) formulated feed after 1, 4, 8, 12, and 16 wk (Experiment 3).

About 30 days after the initial inoculation with the cultured diatom *Navicula cf. jeffreyi* this species out-competed the naturally developing diatom species *Nitzschia* sp., which dominated at the start of the experiment. This demonstrates that the species composition of a biofilm can be altered by inoculation of a cultured diatom. However, if competitive species are present at the time of inoculation, this process might take some time.

In this study, growth rates of $40 \mu\text{m day}^{-1}$ were recorded on a

TABLE 6.

Average cover of *Ulvella lens* (%) and cell density of *Navicula cf. jeffreyi* (cells cm^{-2}) after 1, 2, 4, 6, 8, 10, 12, and 14 weeks.

Week	<i>Ulvella lens</i>	<i>Navicula cf. jeffreyi</i>
1	55	297,890
2	51	110,443
4	36	72,957
6	15	20,596
8	11	11,783
New plates		
10	62	485,126
12	34	57,549
14	28	117,549

TABLE 7.

Proximate composition of the juvenile diets (% \pm S.E., $n = 3$).

	<i>U. lens</i>	<i>N. cf. jeffreyi</i>	Formulated feed
Protein	34.3 ± 1.6	25.1 ± 2.6	28.5 ± 1.1
Carbohydrates	16.9 ± 1.4	16.3 ± 2.2	51.7 ± 1.7
Fat	5.3 ± 1.6	6.7 ± 0.7	4.1 ± 0.2

combined diet of *U. lens* and *N. cf. jeffreyi* until juveniles reached 2 mm in shell length and $70 \mu\text{m day}^{-1}$ until juveniles reached 4 mm in shell length, which compares favorably to other studies reviewed in Kawamura et al. (1998). Although diatoms are a good food source for young post larvae (Daume et al. 2000), *U. lens* may prove to be just as nutritious as diatoms; however smaller juveniles may not be able to graze on it until they reach a certain size. In this study we provide some evidence that juveniles of 2–3 mm in shell length (between 44 and 58 days after larval release) are able to actively remove *U. lens* from the plates and access it as a food source. This was evident by a sharp decline in percentage cover of *U. lens* between these two measurements points. The cell density of the cultured diatom *N. cf. jeffreyi* was low at the start of the experiment, which may have contributed to the lower growth-rate of the abalone at this stage. However, it is more likely that higher growth-rates are observed towards the end of the trial because juveniles are able to access *U. lens* as a food source. As abalone grow, not only does their ability to access food change usually in accordance to their mouth size (Fleming et al. 1996), but also the apparent efficiency of the animal's radula. The radula undergoes morphologic changes during the animal's development and these changes may be linked to a shift in feeding habits from microalgae to macroalgae (Kawamura et al. 2001). The teeth may function as scoops when postlarvae are less than 1 mm in shell length whereas postlarvae larger than 1 mm may be able to scrape and feed on larger particles (Roberts et al. 1999a).

During the juvenile phase, growth of wild *H. laevigata* has been found to be linear (Shepherd 1988) and size variability should remain constant. However, at 44 days after larval release, we observed a clear shift in diet, which caused an increase in growth of the larger juveniles. This resulted in a greater variability (SD) and larger spread (IQD), and change from a uni-modal to a multimodal distribution. Another distinctive increase in SD and IQD was observed between 3 and 16 days after larval release, which coincides with an increase in growth-rate. This is believed to be a shift in nutrition from absorption of yolk reserves to efficient exogenous feeding (Kawamura et al. 1998a). The change in skewness from a longer left tail to a longer right tail after 16 and 44 days indicates that larger juveniles were growing faster. After day 44 the survival stabilized, suggesting that potential stresses associated with morphologic, physiologic, and nutritional changes were overcome.

Early Weaning onto Formulated Feed Versus Algal Feed

Several factors need to be considered when comparing the growth and the survival of juvenile abalone feeding on different food items; the food abundance and availability (e.g., patchiness of the food provided, the effort needed to remove a food item, and biomass ingested per unit grazing effort), and the digestibility and the nutritional value of the food item.

In this study all food was offered in excess but the cell density of *N. cf. jeffreyi* was low at times. Other studies have acknowl-

edged the difficulty to maintain high cell densities in the presence of heavily grazing juveniles (Kawamura et al. 1998b, Roberts et al. 1999b) and the results of this study support their findings. Thus the full potential of diatoms as a food source for juvenile abalone might have been underestimated in this experiment. However, the growth-rates of juveniles were very low despite the high cell density of *N. cf. jeffreyi* at the start of the experiment and when new plates were introduced. In contrast, post larvae 0.4–3.3 mm in shell length show superior growth when feeding on diatom species such as *Navicula* spp. when compared with *U. lens* (Daume et al. 2000). This indicates that diatoms are suitable for small juveniles but *N. cf. jeffreyi* is not sufficient to maintain adequate growth for juveniles larger than 3 mm in shell length. In comparison to the other diets *U. lens* had a relatively high protein content. This could have contributed to the better growth on this diet. Brown & Jeffrey (1995) reported a slightly higher protein concentration, higher fat content, and lower carbohydrate content in *Navicula jeffreyi* compared to the proximate composition of this study. However, it is well known that the biochemical composition of an algal species can change depending on culture conditions such as light intensity and nutrients (Thompson et al. 1993, Fábregas et al. 1996). In our study samples were harvested from the nursery and thus growing conditions were dependent on abalone culture conditions in the outdoor nursery environment that is certainly different from the culture condition in a controlled culture room.

The decline in growth rates, after juveniles were transferred onto new plates, indicates that juveniles might have been stressed and feeding might have slowed due to the handling. However, feeding must have been substantial because there was a subsequent rapid decline in *U. lens* cover and diatom cell density. It may be beneficial to introduce new plates, with the desired algae present, allowing juveniles to move onto the new plates to prevent handling stress. Animals on formulated feed were not transferred at this stage and growth-rates rose slowly.

Growth-rates of juveniles feeding on formulated feed were even lower than on the *N. cf. jeffreyi* at the start and slightly better at the end of the experiment but still remarkably lower than

growth-rates of juveniles feeding on *U. lens*. The growth-rates of juveniles feeding on formulated feed improved towards the end of the trial indicating that the feed may be suitable for juveniles larger than 4 mm in shell length. The formulated feed used in this experiment may not be well matched for the nutritional requirements of small juveniles. The formulated diet was lower in protein and higher in carbohydrates than *U. lens*. Unless a suitable formulated feed is found, which can match growth-rates achieved with the macroalga *U. lens*, we suggest keeping animals on plates colonized by *U. lens* as long as possible. However, the size variability was particularly high in the juvenile population fed on *N. cf. jeffreyi* followed by *U. lens* towards the end of the 16-wk experiment. In this study we provided initial evidence that juveniles feeding on natural food showed higher size variability than juveniles feeding on formulated feed. Feeding formulated diets instead of natural diets such as *N. cf. jeffreyi* may provide a more uniform supply of nutrition throughout the nursery phase (independent of season), growing conditions, and size of the animals; and may ultimately lead to higher growth rates and lower mortality and size variability if the food can be improved. However, culturing *U. lens* as a food source may prove to be more cost effective because there is only a small cost involved in culture set-up and fertilizer. In addition, once *U. lens* is in the nursery system it regenerates *in situ* and no costly algal culture is required. At the present stage we regard *U. lens* as the preferred food species for juveniles >3 mm in shell length, because of the ease with which *U. lens* can be cultured in the nursery and superior growth achieved on this alga.

ACKNOWLEDGMENTS

The authors thank the staff for providing the infrastructure, the abalone larvae, and diatom cultures to inoculate the tanks. They also thank Dr. Rob Day for providing statistical advice and Dr. Greg Maguire and Andrew Hancock for useful comments to improve the manuscript. Great Southern Marine Hatcheries in Albany Western Australia, hosted this experiment. Fisheries Research and Development Corporation supported this study as part of a larger program (FRDC 98/306).

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UTILIZATION OF DIETARY PROTEIN, LIPID, AND CARBOHYDRATE BY ABALONE *HALIOTIS DISCUS HANNAI*: A REVIEW

SANG-MIN LEE

Faculty of Marine Bioscience and Technology, Kangnung National University,
Gangneung 210-702, Korea

ABSTRACT Feeding *Haliotis discus hannai* depends mainly on the use of macroalgae such as *Undaria* sp. or *Laminaria* sp., although the species is an important mariculture shellfish in Korea. Research on the utilization of protein, lipid, and carbohydrate in formulated diets by *H. discus hannai* has been conducted, and the results have been reviewed. *H. discus hannai* fed a formulated diet showed better growth performance than those fed a macroalgae based-diet. Casein, fish meal, soybean meal, cottonseed meal, and wheat germ meal are all good sources of dietary protein for *H. discus hannai*. The optimum dietary lipid content for *H. discus hannai* is from 2% to 5%. Marine fish oil, for instance squid liver oil, is a good dietary lipid source for *H. discus hannai*. Oil supplementation in diets is not necessary when fish meal as main protein source is used for *H. discus hannai* diets. Comparative growth rates were observed when *H. discus hannai* were fed diets containing each of the following carbohydrate sources: wheat flour, dextrin, sucrose, potato starch, or as a mixture combined with practical ingredients such as soybean meal, corn gluten meal, cotton seed meal, and wheat flour. These results may be used in developing practical feed formulation and improving aquaculture production of abalone.

KEY WORDS: *Haliotis discus hannai*, formulated diet, protein, lipid, carbohydrate

INTRODUCTION

Haliotis discus hannai is a commercially important shellfish species in Asia, especially, in Korea, China, and Japan. This species has several desirable characteristics for aquaculture including tolerance to low water temperatures, establishment of seedling production, and ability to withstand high stocking density (Kikuchi & Uki 1974, Uki & Kikuchi 1982, Kim et al. 1998). Farming of *H. discus hannai* in Korea has been developed since 1970, and aquaculture production is currently increasing at a rapid rate. It is likely to maintain this increasing trend in the production because of the decrease in wild abalone captures due to overexploitation, water pollution, and other causes. In culturing *H. discus hannai*, juvenile abalone are reared on diatoms cultured on plates until they grow up to about 5-mm shell length and then weaned on to marine macroalgae.

Abalone has a slow and very heterogeneous growth rate. The growth rate of *H. discus hannai* is approximately 1–2 cm in shell length per year depending on environmental conditions such as water temperature and food. The economic success of abalone aquaculture depends in part on the abalone growth during culture. Nutritional quality of feed is critical, among many factors affecting growth and survival of animals. In the wild, *H. discus hannai* eat mainly macroalgae, such as *Undaria* and *Laminaria*, which are predominated in Korea. It would be expensive to maintain or to culture an adequate supply of live algae. Lee et al. (1997) investigated the effects of experimental formulated diets, commercial diets, and *Undaria* (12.8% crude protein, 1.0% crude lipid) on growth and body composition of *H. discus hannai*. Experimental diets were formulated to contain 34.1% crude protein and 7.5% crude lipid. The abalone fed the experimental formulated diet and several commercial diets showed better survival and growth rate (Fig. 1) than those fed *Undaria*. Similar results for *H. discus hannai* and *H. fulgens* have also been observed in other studies (Nie et al. 1986, Viana et al. 1993). For further expansion of abalone farming, use of formulated artificial feeds is desirable to ensure a better growth.

Development of nutritionally balanced and cost-effective feeds is dependent on information on the nutritional requirements and feed utilization of the species. To date, nutrition research on *H. discus hannai* has identified dietary requirements of protein (Uki et al. 1986a, Mai et al. 1995a), lipids, and essential fatty acids (Uki et al. 1986b, Mai et al. 1995b, Mai et al. 1996), vitamins (Tan & Mai 2001), and minerals (Tan et al. 2001) for normal growth. Other studies have also been conducted to investigate the utilization of dietary sources for protein (Uki et al. 1985, Lee et al. 1998a, Lee et al. 1999a), lipid (Lee & Park 1998), carbohydrate (Lee et al. 1998b), and additives (Lee et al. 1998c, Lee et al. 1999b, Lee et al. 2000, Lim & Lee 2003) in the same species. Based on the nutritional information available, practical feed formulation (Lee 1998) has been studied to improve aquaculture production of *H. discus hannai*. The results of the research on the utilization of dietary protein, lipid, and carbohydrate in *H. discus hannai* are reviewed in this study.

PROTEIN

In nutritional studies on the animals, determination of their dietary protein requirement is generally considered as a primary step. Proteins are not only the major constituent of animal's bodies, but also function as enzymes and hormones. Therefore, a continuous supply of proteins with balanced amino acids is required for maintenance and growth of target animal. Without satisfying the protein requirement, adequate growth and health of the animal cannot be ensured. On the other hand, if excessive proteins are provided, some of them are metabolized as energy; thus the most expensive dietary component will not be effectively used. In the study conducted by Uki et al. (1986a) and Mai et al. (1995a) to determine the protein requirements of *H. discus hannai*, diets containing casein or fish meal as the protein sources were fed to abalone initially weighing 3.5 g and 0.4 g, for 40 days and 100 days, respectively. The research conducted by Uki et al. (1986a) showed that growth of *H. discus hannai* fed diets containing 20% to 30% crude protein was better. Mai et al. (1995a) estimated that the optimum crude protein level for growth of *H. discus hannai* was between 25% to 37%. Taylor (1992) and Coote et al. (2000) reported that dietary crude protein requirements of *H. kamtschat-*

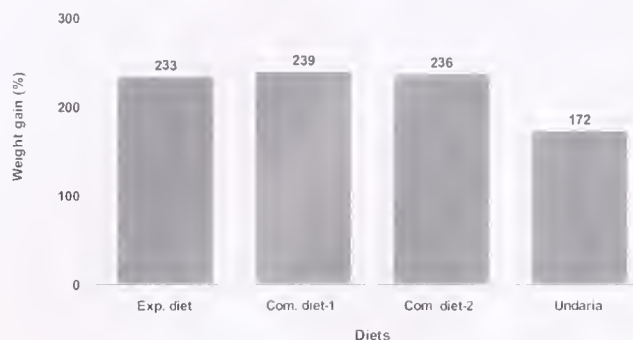


Figure 1. Weight gain [(final weight-initial weight) \times 100/initial weight] of *Haliotis discus hannai* fed the experimental diet, commercial diets and *Undaria* for 17 wk (data from Lee et al. 1997).

kana and *H. laevigata* were 30% and 27%, respectively. *H. midae* exhibited optimum growth rate when crude protein levels are 36% to 47% in the diets (Britz 1996, Sales et al. 2003). These optimum dietary protein levels for abalone are lower than those for most carnivorous and omnivorous fish and similar to those for several herbivorous fresh water fish, such as common carp and Nile tilapia (NRC, 1993). The reason for the different crude protein requirement between studies, even though the same abalone species were used and the rough requirement ranges reported as compared with other fish, is not clear but may be due to differences in abalone size, feeding trial periods, digestible protein to energy ratios, essential amino acid pattern in the diets, culture surroundings, or statistical analysis methods used in the studies. Differences in leaching rates of nutrients from the diets into water could also have resulted in the differences between the protein requirement studies.

Besides biologic protein requirements, economics, and culture strategy need to be considered in formulating practical feeds. This seems to be of primary importance for abalone farmers who want to grow their abalone to marketable size. In this respect, recent experience suggests that it might be necessary to include more than 20% crude protein in the formulation of the practical feeds for *H. discus hannai* to obtain good growth (Lee et al. 1998a). Furthermore, the dietary protein level should be higher to compensate essential amino acid imbalance (NRC 1993, Sales & Britz 2001) when high quality protein in diets is replaced by protein from byproducts without fine tuning of amino acid balance and poor digestibility of those substitutes. The formulation of artificial diets should be based on the availability of the nutrients in the ingredients used and the animal's requirements for those nutrients. Among dietary ingredients, the protein source is important, because protein is much more expensive than lipid or carbohydrate, and the growth of animal could be more influenced by availability of dietary protein sources. Thus, high-quality proteins are necessary for a good artificial feed for abalone. Uki et al. (1985) reported that casein was the best protein source for use in *H. discus hannai* diets. In addition, results conducted by Viana et al. (1993) showed that each of casein and fish meal was equally good protein source for growth of *H. fulgens*. Britz (1996) reported that low-temperature-processed fish meal and *Spirulina* were the most suitable candidates for primary protein sources in formulated diets for *H. midae*. These protein sources are expensive to use in the practical feeds, thus partial or total replacement of casein or fish meal by low-cost practical alternate protein sources would be promising for the economic advantage. An 18-wk growth trial was conducted by Lee et al. (1998a) to evaluate the practical dietary protein

sources for juvenile *H. discus hannai*. Three replicate groups of *H. discus hannai* averaging 110 ± 7 mg were fed one of ten diets containing casein, white fish meal (WFM), meat meal (MM), feather meal (FM), blood meal (BM), soybean meal (SM), corn gluten meal (CGM), cotton seed meal (CSM), *Undaria* powder (UP), or wheat flour (WF) as a dietary protein source. In addition, growth of abalone fed these dietary protein sources was compared with abalone fed macroalgae such as *Undaria* or *Laminaria*. Weight gain (Fig. 2) of *H. discus hannai* fed the diets containing casein, WFM, SM, CSM, or UP was significantly higher than those of abalone fed the other diets. The weight gain of abalone fed the FM, BM, CGM, or macroalgae diets was significantly lower than that of abalone fed the other diets. Lee et al. (1999a) also investigated the utilization of wheat germ meal (WGM) as a protein source replacing fish meal and soybean meal in formulated diet for juvenile *H. discus hannai*. Survival, weight gain, and shell growth of the abalone fed the diets containing 10% to 30% WGM were not different to those of abalone fed the control diet. It is concluded that WFM, SM, CSM, UP, and WGM can be used as good sources of protein in formulated diet for *H. discus hannai*.

It was suggested that a diet containing plant protein sources in combination with animal protein sources produced a better growth of abalone (*H. fulgens* and *H. asinina*) compared with diets containing plant protein sources alone (Guzman & Viana 1998, Bautista-Teruel et al. 2003). The good growth of abalone fed diets containing combination of plant and animal protein sources could have partly been attributed to the fact that some limiting essential amino acids in plant protein sources might be compensated by animal protein sources. Lee (1998) and Kim et al. (1998) also reported the efficiency of the use of combination of plant and animal protein sources in the practical diets for *H. discus hannai*.

LIPID

Dietary lipids play important roles in providing energy, essential fatty acid (EFA), and fat-soluble nutrients for normal growth of animal. Carnivorous fish have limited ability to use carbohydrates as an energy source, but can efficiently use dietary lipid as high as 10% to 25%. In comparison, almost all the herbivorous fish in-

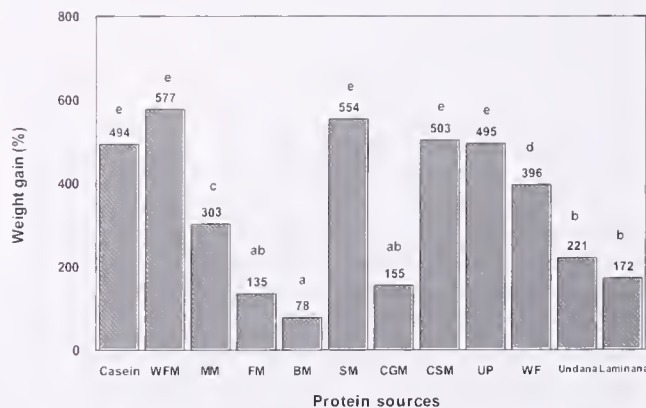


Figure 2. Weight gain [(final weight-initial weight) \times 100/initial weight] of *Haliotis discus hannai* fed the diets containing various protein sources and macroalgae (*Undaria* and *Laminaria*) for 17 wk. Data with different letters were reported as significantly different using ANOVA and a multiple range test with $P < 0.05$ (data from Lee et al. 1998a).

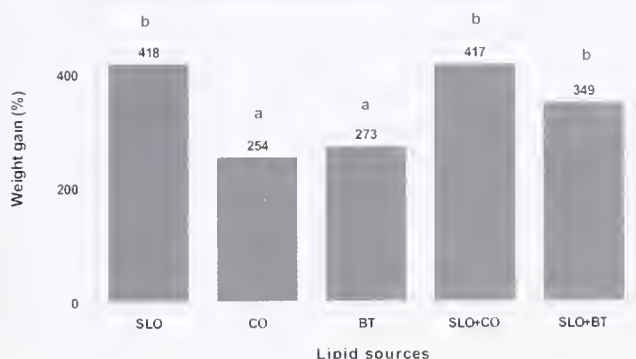


Figure 3. Weight gain [(final weight-initial weight) \times 100/initial weight] of *Haliotis discus hannai* fed the diets containing various lipid sources for 20 wk. Data with different letters were reported as significantly different using ANOVA and a multiple range test with $P < 0.05$ (data from Lee & Park 1998).

cluding abalone can efficiently use carbohydrates as an energy source, but cannot use high lipid levels (Lee et al. 1998b). Mai et al. (1995b) reported an optimum lipid requirement in a formulated diet for *H. discus hannai* to be about 3% to 7%.

EFA requirements in fish differ among species (NRC 1993). Fresh water fish require 18:3n-3 and/or 18:2n-6, whereas marine fish require n-3 highly unsaturated fatty acids (n-3HUFA) such as EPA and DHA. The EFA requirement of *H. discus hannai* has been studied by Uki et al. (1986b). The study showed that *H. discus hannai* requires n-3 and n-6 HUFA as EFA, and the n-3HUFA requirement was about 1% in a diet containing 5% lipid. Experiments were carried out by Lee and Park (1998) to evaluate the effects of lipid source in formulated diets on the growth and body composition of juvenile *H. discus hannai*. Three replicate groups of *H. discus hannai* averaging 151 ± 5 mg were fed the casein-based diets containing 5% squid liver oil (SO), 5% corn oil (CO), 5% beef tallow (BT), 2.5% SO + 2.5% CO, and 2.5% SO + 2.5% BT for 20 wk. Weight gain (Fig. 3) and shell growth of *H. discus hannai* fed the diets containing SO, SO + CO or SO + BT were significantly higher than those of abalone fed the diets containing CO or BT alone. These data indicate that SO is a good dietary lipid source for juvenile *H. discus hannai*. Oil supplement in diets is not necessary when fish meal is used as the main protein source (Lee & Park 1998). Considering the results (Uki et al. 1986b, Mai et al. 1995b, Lee & Park 1998), 2% to 5% crude lipid may be recommended for practical *H. discus hannai* diets, when EFA requirement is satisfied with marine fish oils rich in n-3HUFA.

CARBOHYDRATE

Feed costs in aquaculture can be minimized by optimal use of low-cost energy sources such as carbohydrates. The ability of fish to use different carbohydrate sources and levels differs among species (Wilson, 1994). The utilization of dietary carbohydrates in aquatic animals seems to be related to their digestive and meta-

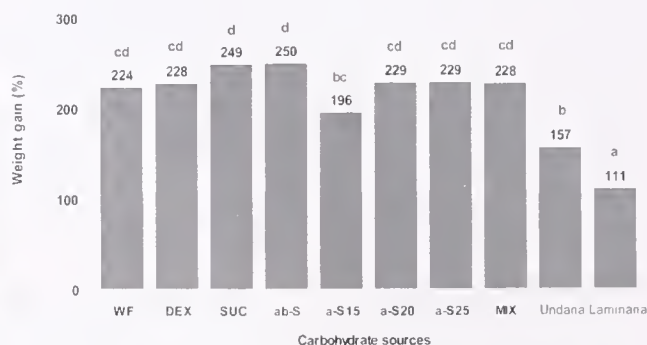


Figure 4. Weight gain [(final weight-initial weight) \times 100/initial weight] of *Haliotis discus hannai* fed the diets containing various carbohydrate sources and macroalgae (*Undaria* and *Laminaria*) for 20 wk. Data with different letters were reported as significantly different using ANOVA and a multiple range test with $P < 0.05$ (data from Lee et al. 1998b).

bolic systems adapted to different aquatic environments and to dietary carbohydrate level and complexity (Walton & Cowey 1982). Carbohydrate utilization by most carnivorous fish is lower than that of herbivorous and omnivorous fish. The growth of fish is reduced when fed carbohydrate-deficient diets because other nutrients such as protein are catabolized for energy and to provide metabolic intermediates for the synthesis of other biologically important compounds (Wilson 1994). A 20-wk growth trial was conducted by Lee et al. (1998b) to investigate the utilization of carbohydrate sources by juvenile *H. discus hannai*. Four replicate groups of *H. discus hannai* averaging 125 ± 1.1 mg were fed one of eight diets containing 24.2% wheat flour (WF), 20% dextrin (DEX), 20% sucrose (SUC), 10% α -potato starch + 10% β -potato starch (ab-S), 15% α -potato starch (a-S15), 20% α -potato starch (a-S20), 25% α -potato starch (a-S25), or mixture of carbohydrates (MIX) with practical ingredients such as soybean meal, corn gluten meal, cotton seed meal, and wheat flour. These formulated diets were compared against *Undaria* or *Laminaria*. Survival, weight gain (Fig. 4) and shell growth of *H. discus hannai* were not significantly affected by the different dietary carbohydrate sources, although those fed the a-S15 diet had slightly lower weight gain than those fed the other formulated diets. *H. discus hannai* fed *Laminaria* had the lowest weight gain, followed by those fed the *Undaria*. These data indicate that *H. discus hannai* were able to efficiently use the carbohydrate sources used in the study. The lower growth rate of *H. discus hannai* fed the a-S15 diet may be due to its high lipid content (7.5%) compared with those (3.1% to 5.3%) of other diets. Research on the digestive enzymes of *Haliotis* spp. has shown that abalone have high protease, amylase, cellulase, and alginase, but low lipases (Oshima 1931, McLean 1970, Cho et al. 1983, Nakagawa & Nagayama 1988, Gomez-Pinchetti & Garcia-Reina 1993, Britz et al. 1994). These investigations indicate that *H. discus hannai* use carbohydrate more efficiently than lipids as an energy source.

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AMINOPEPTIDASE REACTIVITY IN THE DIGESTIVE TRACT OF ADULT ABALONE *HALIOTIS ASININA* LINNAEUS

PORNCHARN SAITONGDEE,¹ PORNRUT RABINTOSSAPORN,¹ PRAPEE SRETARUGSA,¹
TANES POOMTONG,² AND PRASERT SOBHON^{1,*}

¹Anatomy Department, Faculty of Science, Mahidol University, RamaVI Road, Bangkok 10400, Thailand; ²The Coastal Aquaculture Development Center, Department of Fisheries, Ministry of Agriculture and Cooperatives, Klong Wan, Prachaubkirikhun 77000, Thailand

ABSTRACT Histologically, the epithelium of the digestive tract is pseudostratified columnar type, which is composed of 3 kinds of cells (i.e., granulated cells, nongranulated cells, and mucus cells). The subsets of these three types of cells vary from region to region. By an enzyme-histochemical method, leucine aminopeptidase reactivity was localized principally within the cytoplasm of granulated cells in the buccal cavity, esophagus, esophageal pouch, hepatopancreas, and the third intestinal region, which implies that these sites are responsible for the synthesis and secretion of this peptidase for the digestion of proteinaceous nutrients.

KEY WORDS: *Haliotis asinina*, abalone, digestive tract, aminopeptidase

INTRODUCTION

The abalone *Haliotis asinina* is considered the most economically important abalone among the three species found along the coastal water of Thailand (Nateewathana & Bussawarit 1988) because of its good taste, high proportion of flesh, and the fastest growth rate. Due to increased demand, the collection of these abalone from natural habitat could not keep pace. To increase the abalone stocks and yields, the animals have been cultured and fed artificial diets for enhancing the growth rate (Serviere-Zaragoza et al. 1997). The rate of growth and nutritional composition of abalone were significantly affected by the level of proteins in the diet (Britz & Hecht 1997), which could be increased up to 30% (Fallu 1991). This high protein content could be broken down by proteolytic enzymes in the digestive tract of abalone, even though abalone are considered to be herbivorous animals that feed on mainly macroalgae, which are richer in carbohydrates. One possible protease inducible in the abalone is aminopeptidase, which is a family of zinc-dependent enzyme that catalyzes the hydrolysis of amino acid residues at the amino terminus of peptide substrates (Acosta et al. 1998). The source of this enzyme is most likely the epithelium of the digestive tract. The epithelial cells of the digestive tract of gastropods have been classified into 3 cell types and termed by different names; for example, columnar storage cell, secretory cell and mucus-producing cell (Tiebskorn & Kunast 1990), nonciliated, ciliated, and glandular cell (Leal-Zanchet 1998, Roldan & Garcia-Corrales 1988), Campbell (1965) and Chitramvong et al. (2000) have studied the abalone and also classified these epithelial cells into 3 types, which include pigmented cell, secretory cell, and mucus cell. The aim of this study is to investigate the presence of aminopeptidase in epithelial cells of some parts of the digestive tract of *H. asinina* by an enzyme-histochemical method.

MATERIALS AND METHODS

Collection of Abalone Specimens

Adult abalone *H. asinina* (age >16 mo old) of both sexes were obtained from the Coastal Aquaculture Development Center, De-

partment of Fisheries, Prachuabkirikan province, Thailand. The animals were reared in the raceways of concrete tanks, which were well flushed with mechanically circulated seawater and air delivery system to maintain controlled environment. Abalone were fed with a diet of macroalgae (*Gracilaria* spp. and *Laminaria* spp.) supplemented with artificial food, and kept under normal daylight cycle.

Burstone and Folk Method for Detecting Aminopeptidase

Abalone with body weights of 36.5 ± 2 g were anesthetized with 5% magnesium chloride for about 30 min. The shells (average size of 6.5 cm \times 2.8 cm) were removed and digestive organs were dissected out and diced into small pieces before being embedded in the cryo-supporting medium. Frozen specimens were sectioned at 5- μ m thick. The cryosections were fixed in two changes of chloroform for 4 min each, then hydrated in graded series of acetone, and finally in distilled water. The sections were incubated in a substrate solution containing the mixture of L-leucyl-b-naphthylamide, distilled water, tris buffer and garnet GBC for 1h at 37°C, then in tap water for 5 min and counterstained by Hematoxylin. After the final run through tap water for 10 min, the sections were mounted on glass slides in buffered glycerol (Burstone & Folk 1956).

Histology of the Digestive Tract

To identify the cell types and characterize the cellular details for the enzyme localization, the histology of the abalone digestive tract was also studied from paraffin and semi thin plastic sections by preparing the specimens as follows.

For paraffin sections, various parts of the digestive tract were cut and fixed in the fixative of Bouin at 4°C overnight. Specimens were washed in 70% ethyl alcohol for removal of the fixative. Then, they were dehydrated through a graded series of ethyl alcohol (70% to 100%) for 1–2 h each, cleared with dioxane, infiltrated and embedded in paraffin wax, sectioned at 5 μ m thick, and finally stained with Hematoxylin-Eosin (H&E) or Hematoxylin-PAS (H&PAS). The specimens were observed and evaluated for the characteristics of each cell type under microscope equipped with a digital camera.

For semithin plastic sections, the corresponding parts of the digestive tract were fixed in a solution of 4% glutaraldehyde and

*Corresponding author. Fax: +662-354-7168; E-mail: sepso@mahidol.ac.th

2% paraformaldehyde in 0.1 M Millonig buffer at 4°C overnight. After washing with buffer, the samples were postfixed in 1% osmium tetroxide in 0.1 M Millonig buffer at 4°C for 1 h, and then dehydrated in graded series of ethanol and pure propylene oxide. After that the specimens were infiltrated and embedded in Araldite 502 resin, and finally polymerized at 60°C for 24 h. Blocks of plastic embedded specimens were sectioned at 1 µm. These semithin sections were stained with 1% methylene blue, and then observed under microscope equipped with a digital camera.

RESULTS

Histology and Aminopeptidase Reactivity

The Buccal Cavity

The epithelium of buccal cavity consists of 3 cell types, the tall granulated columnar cells bearing microvilli; which appear as a "brush border" under the light microscope, the nongranulated cells, and the mucus cells having a "goblet" appearance; which are widely scattered in the epithelium (Fig. 1a). The granulated cells show red orange product in the apical cytoplasm overlapping on the granulated area (Fig. 1c), indicating the presence of aminopeptidase in this area of the cytoplasm, and at the luminal surface, whereas nongranulated and mucus cells are not stained. The control section does not show the presence of aminopeptidase (Fig. 1b).

The Esophagus

The epithelium of the esophagus contains 4 cell types: 2 types of granulated columnar cells, nongranulated columnar cells, and mucus cells (Fig. 1d to f). The two types of granulated columnar cells are distinguished by the presence of different size granules and color (i.e., in H&E stain type 1 contains small brownish granules whereas type 2 contains large reddish eosinophilic granules, whereas in semithin section all the granules appear bluish with different intensities (Fig. 1d to f)). The granulated cells with large reddish granules, nongranulated cells, and the mucus cells do not show aminopeptidase reactivity, whereas the granulated cells with small brownish granules show intense aminopeptidase reactivity in the granules (Fig. 1h). The control section does not show red product (Fig. 1g).

The Crop

The epithelium of crop appears to be pseudostratified columnar epithelium that is composed of only one-cell type (i.e., the tall columnar cells, bearing a brush border). These cells also contain small clear mucin granules that are tightly packed together in both the apical and basal cytoplasm (Fig. 1i). This cell type shows only weak reactivity at its luminal surface but not in the cytoplasm (Fig. 1k), hence probably does not contain aminopeptidase enzyme.

The Stomach

The stomach epithelium comprises 3 cell types: mucus cell, tall granulated columnar cells with fine granules in the apical cytoplasm, and nongranulated columnar cells. The epithelium is covered by a thick layer of PAS-positive gastric shield (Fig. 1l). The intense aminopeptidase reactivity is present in the gastric shield overlying the epithelium but absent in the epithelial cells' cyto-

plasm. (Fig. 1n). The control section shows negative reaction (Fig. 1m).

The Intestine

The intestinal epithelium contains four types of columnar epithelial cells. The former group comprises two types of granulated cells with different characteristics of granules (i.e., the first type of granulated cell appears to be similar to those found in the stomach whereas the second type possesses larger granules with varying density (Fig. 2a, b, c)). There is one type of nongranulated cell (Fig. 2b), and one type of mucus cell appearing similar to those in the crop (Fig. 2a). All two types of granulated cells show intense aminopeptidase reactivity in their cytoplasm (Fig. 2d, g). These four cell types occupy almost the entire epithelium except at the two prominent longitudinal folds, which contain only nongranulated cells that are devoid of aminopeptidase reactivity (Fig. 2d, e). The mucus cells, which are dispersed in the epithelium do not show any aminopeptidase reactivity either. The control section does not show any reaction product (Fig. 2f).

Hepatopancreas

The hepatopancreas comprises a large number of acini (Fig. 2h). The apical part of the acinar cells near the lumen exhibits intense aminopeptidase reactivity (Fig. 2j). The control section shows no reaction of aminopeptidase (Fig. 2i).

DISCUSSION

In this study epithelial cells of the abalone digestive tract is classified into 3 types. The granulated cells (GC) are so called because they contain dense granules (serous type) with different sizes. This cell type could correspond to secretory cells as reported by Campbell (1965) and Chitramvong et al. (2000). In contrast to these earlier works we could identify at least four subtypes of these cells in various parts of the digestive tract because of their well preserved characteristics in semithin plastic sections. All GC except those in the stomach exhibit the aminopeptidase activity, which overlaps on the granulated area of the cytoplasm. There is only one type of nongranulated cell (NC), which does not exhibit aminopeptidase activity. There are at least 2 types of mucus cells (MC), in contrast to only one type of mucin producing cell reported by Campbell (1965) and Chitramvong et al. (2000). The first type of MC appears like goblet cells containing large mucin granules in the apical part, which tend to be dissolved away as shown in buccal cavity, esophagus, and stomach; whereas the second type is usually loaded with small tightly packed mucin granules as shown in the crop and intestine. Again the identification of these two subtypes of mucus cells is possible because of the use of plastic embedment. The presence and distribution of proteolytic enzyme was studied by detecting aminopeptidase (AP) reaction in different regions of the alimentary tract, from the buccal region to the anus. Almost all parts of the digestive tract show positive AP activities restricted at the brush border of the epithelial cells or in the lumen. The distinct appearance of AP reactivity was only observed in the cytoplasm of the granulated cells of buccal cavity, esophagus, esophageal pouch, as well as intestine. In addition, the intense enzyme reactivity was also detected in the acinar cells of the hepatopancreas. Hence these cells are producing zymogen granules that may contain aminopeptidase. The results agree with the work of Serviere-Zaragoza et al. (1997) who detected the pro-

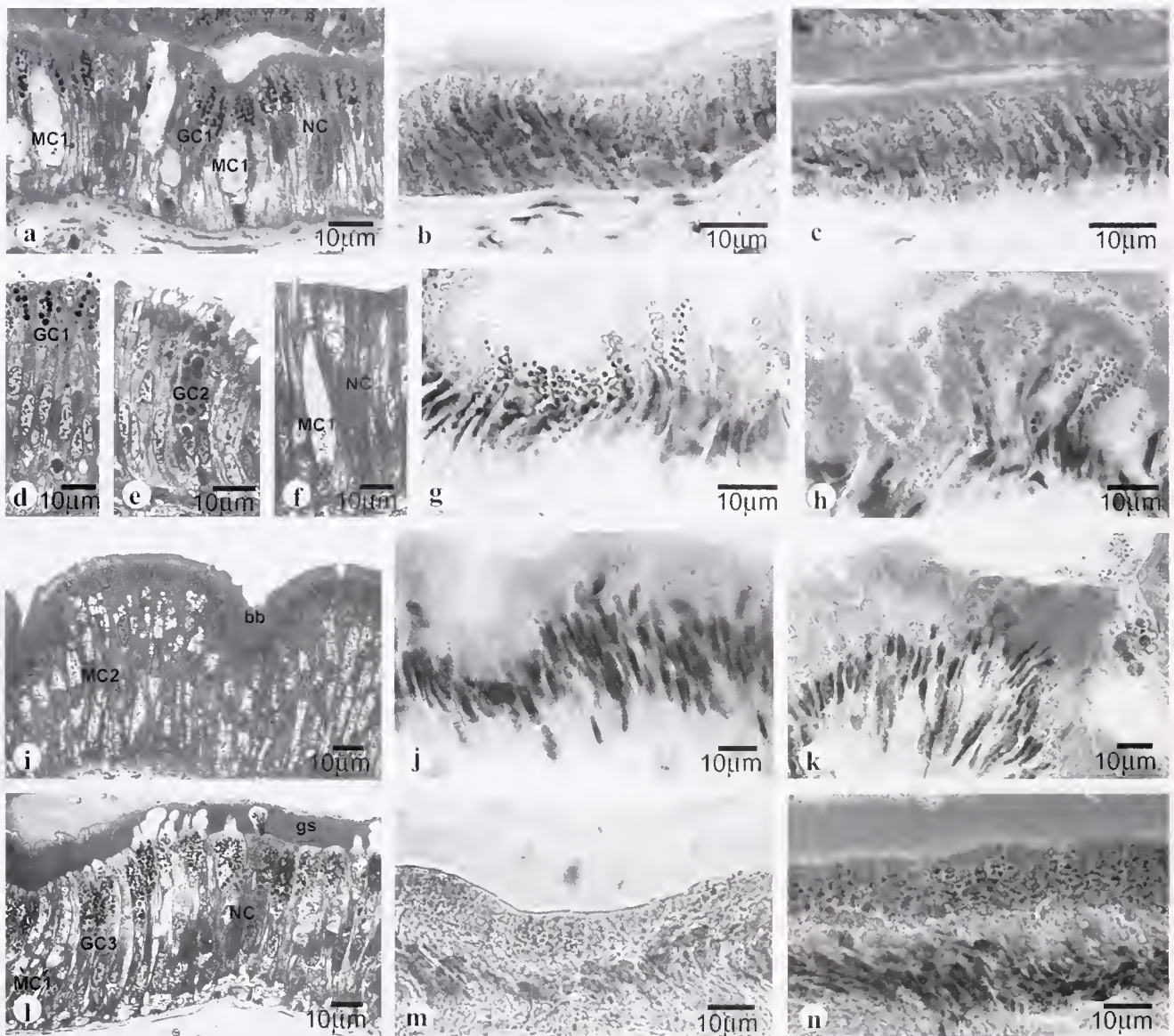


Figure 1. The epithelium of various parts of *H. asinina* digestive tract. Left column: semithin sections stained with methylene blue. Middle column: The control sections for aminopeptidase reaction. Right column: The cryosections exhibiting the presence of aminopeptidase (AP) reactivity. (a.) A semithin section of buccal cavity stained with methylene blue showing tall granulated columnar cells (GC1) bearing microvilli, nongranulated cell (NC), and mucus cells (MC1). (b.) A control cryosection showing no AP reactivity. (c.) A cryosection showing moderate AP reactivity at the luminal surface of the granulated columnar cell. (d to f.) Semithin sections of esophagus showing 4 cell types including granulated columnar cells with small size granules (GC1) and granulated cell with large size granules (GC2), nongranulated cells (NC) and mucus cells (MC1). (g.) The absence of the AP reactivity in the control cryosection. (h.) An intense AP reactivity in the granulated columnar cells. (i.) A semithin section of crop showing the tall columnar cells bearing brush border and containing small clear mucin granules (MC2) in the cytoplasm. (j.) The control section showing no AP reactivity. (k.) AP reactivity is localized on the luminal surface but not in epithelial cell cytoplasm. (l.) A semithin section of the stomach showing 3 types of epithelium cells (i.e., mucus cell [MC1], tall granulated columnar cells [GC3] containing fine granules at the apical part, and nongranulated cells [NC]). Note the presence of a thick gastric shield (gs) covering the luminal surface of the epithelium. (m.) Control cryosection showing no reactivity. (n.) Cryosection showing moderate aminopeptidase reactivity at gastric shield but not in the epithelial cells.

teolytic activity in the hepatopancreas, crop and stomach content, and intestine and rectal fluid of the blue abalone, *H. fulgens*. The proteolytic activity observed in the intestinal and rectal fluid has optimal pH at alkaline range, whereas those from the hepatopancreas, crop, and stomach content were at acid pH. In the hepatopancreas, many proteases (three types of hydrolases and a carboxypeptidase) were detected by enzyme assays in *H. midae* (Erasmus et al. 1997, Hernandez-Santoyo et al. 1998). Moreover, trypsin and

chymotrypsin activities were also detected in the intestine and rectum by using specific-synthetic substrates and inhibitors in kinetic and electrophoresis assays (Groppe & Morse 1993, Serviere-Zaragoza et al. 1997). The AP reactivity recognized in other parts of the digestive tract observed in the present study could possibly be the result of the released enzyme being mixed with the nutrients passing through these regions of the digestive tract rather than the enzyme being synthesized by epithelial cells of these regions. This

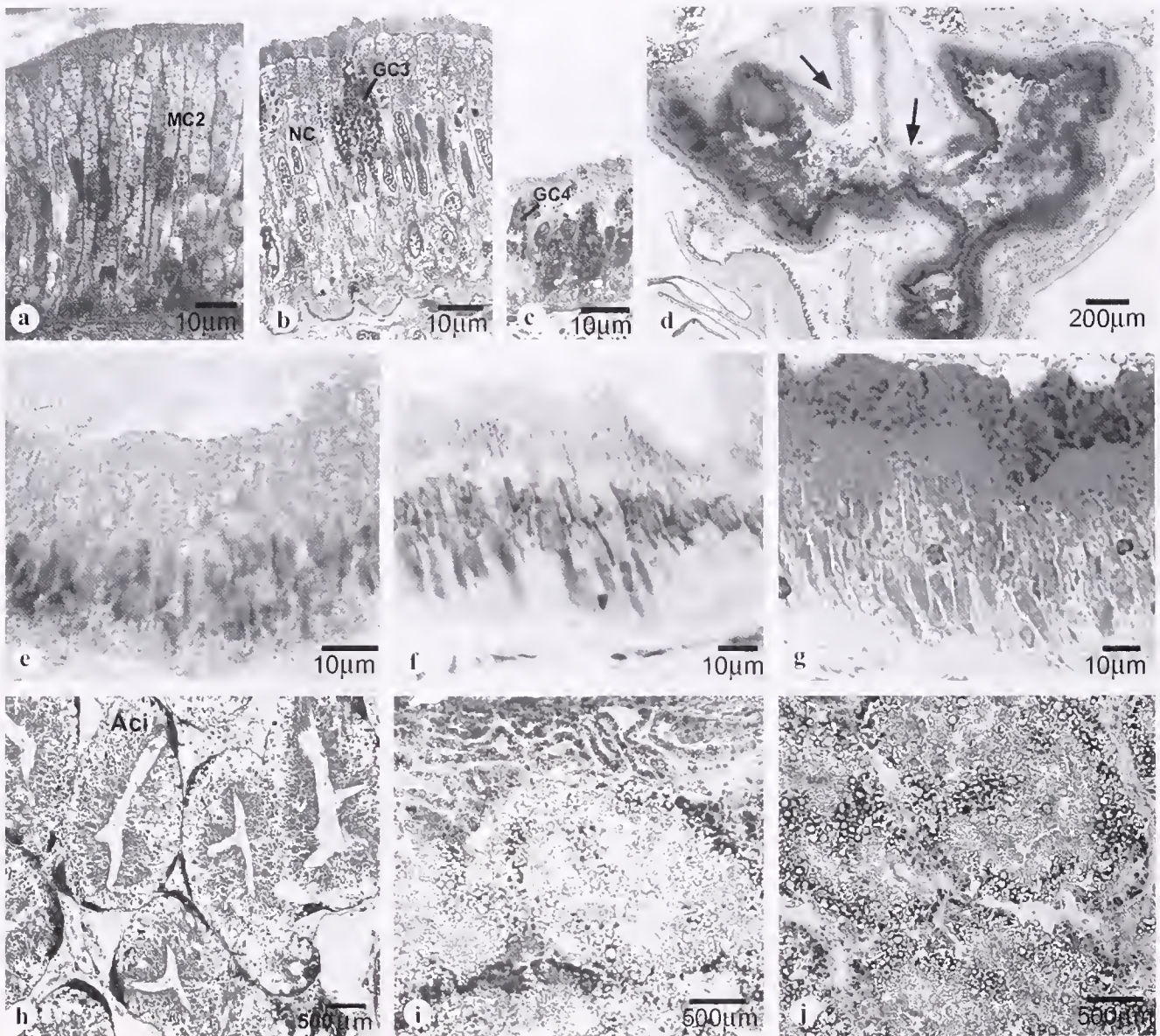


Figure 2. (a to c.) Semithin sections of the intestine showing 4 types of columnar epithelial cells (i.e., granulated cells with small granules [GC3], with large variable density granules [GC4] in the apical cytoplasm, mucous cells [MC2], and nongranulated cells [NC]). (d.) A low magnification of the intestine showing intense AP reactivity in most epithelial area except at the two prominent longitudinal folds (typhlosole) (arrows). (e.) Higher magnification of the area of typhlosole in d showing no AP reaction. (f.) The control cryosection of intestine showing the absent of AP reactivity. (g.) The higher magnification of the intestinal epithelium in d showing intense AP reactivity in their cytoplasm. (h.) Paraffin section of the hepatopancreas stained with H&E, showing many acini (Aci). (i.) The control cryosection showing no AP reactivity. (j.) The cryosection of hepatopancreas showing intensely AP reactivity near the lumen.

could explain the weak AP activity at the luminal surface of the crop, the first and second intestinal parts, and in the gastric shield of the stomach.

The absence of AP activity on the secondary foldings in the crop epithelium indicates that this organ may be the main part for food absorption rather than secreting the enzymes. Our observation is in agreement with Mclean (1970) who found, by using autoradiography with ^{14}C -labeled algae, that in *H. rufescens* the crop is the major absorptive area. This author also demonstrated that the crop epithelium is permeable to phenylalanine and glucose. However, enzymes other than protease may be present in crop, because

high lipase activity was detected in the crop (Mclean 1970). Interestingly, in the intestine there is an area called the longitudinal fold (typhlosole), which does not exhibit any AP activity as most cells are nongranulated, and this structure probably is involved mainly in the absorption, whereas the rest of intestine synthesizes and secretes the protease enzyme.

ACKNOWLEDGMENT

This research was supported financially by the Thailand Research Fund (Senior Research Scholar Fellowship to Prasert Sobhon)

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EFFECTS OF STARVATION ON ENERGY RESERVES IN YOUNG JUVENILES OF ABALONE *HALIOTIS DISCUS HANNAI* INO

SHAOBO DU AND KANGSEN MAI*

The Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, Qingdao 266003, People's Republic of China

ABSTRACT Effects of various degrees of starvation (0-, 5-, 10-, 15-, 20- and 25-day fasted) on tissue protein, lipid, total carbohydrate, glycogen and ash contents, as well as the ratio of soft-tissue to shell weight, were investigated in young juvenile abalone, *Haliotis discus hannai* Ino (initial shell length: 4.8 ± 0.6 mm, initial live weight: 15.0 ± 1.3 mg). Results showed that after 25-day starvation, tissue protein, lipid, total carbohydrate, and ratios of soft-tissue to shell weight decreased significantly, whereas the glycogen level remained constant, and ash content increased significantly. During the 25-day starvation, juvenile abalone lost 23.4% of protein, 19.8% of lipid, and 13.3% of total carbohydrate. The corresponding energy depletions were 226.5, 81.3, and 3.0 kJ/100 g dry body weight, respectively. The variation of energy depletion among the energy components is probably evidence of selective utilization of energy reserves by juvenile abalone, and partly a reflection of the predominance of energy components in juvenile tissues.

KEY WORDS: abalone, *Haliotis discus hannai*, young juvenile, starvation, energy reserves

INTRODUCTION

In abalone hatcheries, benthic diatoms have traditionally been used as initial foods for young juveniles. During the stage when young juveniles are at 5–10 mm of shell length, weaning on to macroalgae or artificial diet, the supply of diatoms has been identified as a major obstacle in abalone nutrition, owing to problems relating to the quality and quantity of diatoms (McCormick & Hahn 1983, Ebert & Houk 1984, Hahn 1989, Knauer et al. 1996). Starvation often occurs when juveniles are not able to ingest sufficient optimal diatoms, which is probably one of the key factors accounting for the high mortality in many abalone hatcheries all over the world.

Starvation is not an uncommon circumstance for marine invertebrates (Carefoot et al. 1993). During starvation, an organism's normal energy mobilization and metabolism is altered, and energy reserves (mainly tissue protein, lipid, and carbohydrate) are catabolized and oxidized to supply basal metabolism. Therefore, starvation must have profound effects on the organism's physiologic condition and tissue chemical content. Many researchers have conducted studies to investigate the effect of starvation on energy reserves in marine gastropods and bivalves. Results show that tissue protein, lipid, and carbohydrate play different roles in different species of marine invertebrates. Most studies on larval bivalves showed that lipid acted as the main energy reserves (Millar & Scott 1967, Corner & Cowey 1968, Holland & Spencer 1973, Whyte et al. 1987). Rodriguez et al. (1990) found that larval oyster, *Ostrea edulis*, consumed protein as the main energy source. In gastropods, available data shows that carbohydrates acted as the main energy reserves (Martin 1961, Goddard & Martin 1966). Carefoot et al. (1993) studied the effect of starvation on tissue glycogen in the northern abalone, *Haliotis kamtschatkana* of ~100–150 g live weight, and results showed that glycogen reserves in the digestive gland and foot muscle were depleted. However, there was no information on the changes of protein and lipid content during starvation.

This study is conducted to investigate the effects of starvation on the energy reserves in young juveniles of *H. discus hannai* at weaning stage, from which we can get some useful information on

the energy metabolism and nutritional requirement of abalone during this stage.

MATERIALS AND METHODS

Young Juvenile Culture and Sample

The experiment was conducted in the Mashan Sea-product abalone hatchery in Shandong Province, People's Republic of China. All the young juveniles used had been settled and grown on diatoms for 2 mo, and showed excellent performance both in growth and survival. At the beginning of the experiment, 150 young juveniles were randomly collected as the sample of Day 0, and initial shell length and live weight were measured. Three PVC hatchery tanks ($50 \times 40 \times 30$ cm) were set up as three replicates. Each tank was stocked with 500 young juveniles on black corrugated plastic plates (45×30 cm), and covered with a 1.5 mm mesh. To minimize the possibility of diatom growth contributing food for the larvae, seawater used was filtered twice with 10- μ m meshes, and the corrugated plastic plates and the tanks were thoroughly scrubbed daily. During the experimental period, water temperature ranged from 21°C to 25°C and the salinity from 31‰ to 33‰. At Day 5 and thereafter, 50 young juveniles from each tank were randomly sampled every 5 days until Day 25, and stored at –20°C for subsequent analyses. Six samples (Days 0, 5, 10, 15, 20 and 25) were achieved altogether.

Methods of Analysis

Samples were freeze-dried, and then soft-tissue was separated from the shell. The contents of protein, lipid, total carbohydrate, glycogen, and ash in soft-tissue were determined, and the ratio of soft-tissue to shell weight of each sample was calculated.

Tissue protein content ($N \times 6.25$) was calculated from the determination of total N by Kjeldahl analysis. Ash content was calculated by gravimetric analysis following loss of mass on ignition at 550°C for 24 h in a muffle furnace. Extraction of lipid was carried out by the method of Bligh & Dyer (1959), and the lipid levels were determined gravimetrically.

Total carbohydrate content was determined by the methods of Dubois et al. (1956), and modified by Towle and Giese (1966). D-glucose was used as a calibration standard. Thirty milligrams of soft-tissue were boiled for 10 min with 3 mL 15% TCA, then the

*Corresponding author. E-mail: kmai@ouc.edu.cn

mixture was centrifuged at 3,500 rpm for 10 min. TCA (15%) was added to the supernatant to the total volume of 10 mL. Two milliliter of this mixture was mixed with 1 mL 5% phenol and 5 mL concentrated sulfuric acid ($\rho = 1.84$). The mixture was allowed to stand for 10 min and then was shaken and placed in a water bath at 30°C for 15 min. The absorbance at 490 nm was measured spectrophotometrically. Total carbohydrate content was calculated as amount of released glucose (mg) per mg soft-tissue $\times 100$.

Glycogen content was measured by the method of Handel (1965), and glycogen was used as a calibration standard. Fifteen milligrams of soft-tissue were boiled with 1 mL 30% KOH for 1 h. The mixture was precipitated with 95% ethanol and glycogen determined by the anthrone method. Tissue glycogen content was calculated as amount of released glycogen (mg) per mg soft-tissue $\times 100$.

Statistical analysis

Data from each sample were subjected to one-way ANOVA and Duncan test to determine difference in means. Prior to analysis, data on percentages were transformed using squared root of arcsine, to make the variance independent of the mean; alpha levels for all tests were set at 0.05. Statistical analysis was performed using Systat package.

RESULTS

Shell length, live weight, and tissue chemical composition of juvenile abalone are shown in Table 1. Shell length maintained almost the same within the first 15 days, then there was a slight increase in shell length. Meanwhile, live weight showed a declining trend. However, these changes in shell length and live weight during the 25-day starvation were not statistically different.

Tissue protein content decreased dramatically from 53.8 to 41.2% during starvation, and there was a significant difference between the batches of Day 0–5 and the batches of Day 10–25 ($P < 0.05$). After Day 10, protein content kept almost constant. The decreased protein content at Day 25 amounts to 23.4% of the initial tissue protein (Day 0).

Tissue lipid content decreased continuously from 11.6% to 9.3% during the starvation period. After Day 15, tissue lipid was significantly lower than the initial value ($P < 0.05$). Lost lipid in the juvenile abalone at Day 25 amounts to 19.8% of the initial content (Day 0).

Total carbohydrate content also declined significantly from 12.9% to 11.2%. In the early period of starvation (from Day 0 to 5), there were no significant changes in total carbohydrate content. At Day 10, however, the total carbohydrate content declined sig-

nificantly ($P < 0.05$), and after that time it remained almost the same. The decline in total carbohydrate by Day 25 amounted to 13.3% of the initial value (Day 0).

A slight declining trend, from 4.8% to 4.6%, was observed in the tissue glycogen during the experimental period, but there was no overall significant difference ($P > 0.05$).

Tissue ash content increased significantly from 12.7% to 24.1% with the prolonged time of starvation ($P < 0.05$). Ash content in larvae of Day 20 and 25 nearly doubled that of Day 0.

The ratio of soft-tissue to shell weight decreased significantly from 0.26 to 0.12 ($P < 0.05$). And the amount declined during Day 0 to Day 10 was larger than that happened in the period from Day 15 to 25.

DISCUSSION

In the present study, almost the same shell length was maintained within the first 15 days, then there was a slight increasing trend, though this change did not have any statistical significance. This means either that the juvenile abalone might be still able to obtain limited nutrients from the environment to support shell growth, or that shell could grow using the limited nutrients stored in abalone tissues. Meanwhile, live weight showed a more obvious decreasing trend during the 25-day starvation, though the change in live weight was not statistically different either. The significant losses of organic matters (protein, lipid, and carbohydrate) in abalone tissues demonstrated that the young juveniles obtained very little nutrients from the environment. The loss of live weight, due to organic matter decrease, was probably compensated by water refilling in tissues. Hence, the results and conclusions in this study were not significantly affected by exogenous nutrients.

The tissue organic matter decreased continuously with the time of starvation suggesting that the energy reserves (protein, lipid, and total carbohydrate) were catabolized and oxidized to supply the energy required for metabolism. The decreases of tissue reserves mainly occurred during the first 15 days (protein), or 20 days (lipid and carbohydrate), and then the declines were at slower and more constant rates. Previous studies also showed that the decline of energy reserves was most rapid during the first few days during starvation in some marine invertebrates (von Brand et al. 1957, Duerr 1965, Carefoot et al. 1993). During the period of starvation, tissue protein, lipid and total carbohydrate content declined significantly, but there was little change in glycogen content. Taking the mean values of protein, lipid, and total carbohydrate content lost by the juveniles after 25 days of starvation, and converting to joule equivalents (Beukema & de Bruin 1979), it is apparent that juvenile abalone obtained more energy from protein

TABLE 1.

Tissue chemical composition in juvenile abalone *Haliotis discus hannai* Ino under different starvation.

	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25
Mean shell length (mm)*	4.79 (0.64)	4.81 (0.57)	4.79 (0.36)	4.81 (0.28)	4.92 (0.19)	5.07 (0.57)
Mean live weight (mg)*	15.0 (1.33)	16.0 (3.18)	14.9 (2.46)	13.0 (1.85)	14.0 (2.04)	13.6 (1.79)
Tissue protein (%)	53.83 ^b (3.42)†	49.98 ^b (1.37)	43.02 ^a (4.18)	41.75 ^a (1.62)	42.03 ^a (2.15)	41.22 ^a (1.87)
Tissue lipid (%)	11.64 ^c (0.54)	11.06 ^{bc} (0.34)	10.65 ^{bc} (0.59)	10.00 ^{ab} (0.21)	9.36 ^a (0.96)	9.33 ^a (0.92)
Total carbohydrate (%)	12.89 ^b (0.22)	12.62 ^b (0.58)	11.52 ^a (0.48)	11.99 ^{ab} (0.44)	11.27 ^a (0.63)	11.17 ^a (0.78)
Tissue glycogen (%)	4.81 (0.27)	4.84 (0.21)	4.83 (0.13)	4.73 (0.35)	4.61 (0.27)	4.66 (0.13)
Ash content (%)	12.69 ^a (1.43)	15.15 ^{ab} (1.70)	16.96 ^{ab} (2.56)	17.75 ^{ab} (0.84)	20.24 ^b (0.93)	24.12 ^b (2.58)
Soft-tissue to shell weight	0.26 ^d (0.025)	0.20 ^c (0.006)	0.17 ^{bc} (0.01)	0.15 ^{ab} (0.015)	0.15 ^{ab} (0.015)	0.12 ^a (0.015)

* Values of shell length and live weight represent mean (SEM), $n = 60$. Other values represent mean (SEM) ($n = 3$).

† Means in each row sharing the same letter are not significantly different based on Duncan test ($P > 0.05$).

(226.5 kJ/100 g dry weight) than lipid (81.3 kJ/100 g dry weight) and total carbohydrate (3.0 kJ/100 g dry weight). The constant content of glycogen indicated either that glycogen was not in great demand or that it was resynthesized from other constituents by glycconeogenesis. Further studies into glycogen metabolism in juvenile abalone should be carried out.

The variation of energy from different substrates probably means that juvenile abalone have selective utilization of energy reserves during starvation. However, the higher energy lost from protein may be partly due to its predominance in young juveniles. There have been different results of energy reserve usage elicited by different researchers, even from the studies on the same species of invertebrates. A number of studies on energy reserves in larval oysters, *Ostrea edulis*, during metamorphosis showed that lipid acted as the major energy supplier (Millar & Scott 1967, Holland & Spencer 1973). Results from Bartlett (1979) and Rodriguez et al. (1990) indicated that in *Ostrea edulis* and *Crassostrea gigas*, tissue protein supplied most of the energy during metamorphosis. The disagreement of these results can possibly be attributed to the different experimental conditions, and the nutritional status, of oyster larvae. Furthermore, Millar & Scott (1967) pointed out that the role of energy reserves in energy metabolism varies with the developmental stages of animals. Results of the present study dif-

fered from that of Carefoot et al. (1993), which showed that tissue glycogen acted as the main energy supplier during starvation. Available evidence suggests that energy metabolism of abalone is based on the utilization of carbohydrates, because their natural diet is rich in carbohydrates (Painter 1983), and similarly, abalone tissue contains rich stores of glycogen (Webber 1970, Knauer et al. 1994). In larval abalone, however, energy metabolism may differ largely from that in the adult. In general, juvenile animals require more protein per unit of body weight because their growth rate is higher than that of older individuals (Mercer et al. 1993). In the wild, larval and juvenile abalone might ingest diatoms with high levels of protein, because the protein content of benthic diatoms ranges widely from 3.5% to 47% (Ansell et al. 1964, Darley 1977). In juvenile abalone, there are higher contents of tissue protein and lipid than in older individuals (Mai et al. 1995), but lower glycogen content. There is probably a relationship between the initial biochemical composition and the role of various energy reserves during starvation.

ACKNOWLEDGMENTS

The authors thank Mashan Sea-product Company for providing the larval abalone and the experimental facilities.

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INFLUENCE OF DIETARY LIPID SOURCES ON GROWTH AND FATTY ACID COMPOSITION OF JUVENILE ABALONE, *HALIOTIS DISCUS HANNAI* INO

WEI XU, KANGSEN MAI,* WENBING ZHANG, ZHIGUO LIUFU, BEIPING TAN, HONGMING MA, AND QINGHUI AI

The Key Laboratory of Mariculture (Education Ministry of China), Ocean University of China, Qingdao 266003, People's Republic of China

ABSTRACT A study was conducted to evaluate the effects of dietary lipid sources on the growth and fatty acid composition of juvenile abalone *Haliotis discus hannai* Ino. Four triplicate groups of juvenile abalone (initial weight: 0.13 ± 0.04 g; initial shell length: 10.23 ± 1.48 mm) were fed with one of four semipurified diets containing 3.5% of dietary lipid from either tripalmitin (TP), soybean oil (SO), linseed oil (LO), or 20:5n-3-enriched fish oil (FO), respectively, and were reared for 120 days in a recirculation water system. Results showed that the growth rate of abalone was significantly affected by dietary lipid sources ($P < 0.05$). The dietary lipid, FO produced the highest weight gain rate (WGR, 413.2%), closely followed by LO (389.0%) and SO (382.4%). These three WGR values were not significantly different to each other. However, abalone fed the TP diet showed a significantly lower WGR (267.3%). Carcass moisture and protein were independent of dietary treatment, but the TP diet resulted in significantly lower carcass lipid. The fatty acid profile in abalone carcass reflected that of dietary lipids, especially the unsaturated fatty acids. It appears that *H. discus hannai* have a capacity to synthesize 20:5n-3, 20:4n-6 and 22:6n-3 from 18-carbon PUFAs and even from 16:0 through elongation and desaturation. However, the capacity seems to be insufficient to support the maximum growth of abalone.

KEY WORDS: *Haliotis discus hannai*, lipid sources, fatty acid composition, growth, feeding and nutrition mollusk

INTRODUCTION

Dietary lipid plays important roles in providing concentrated energy, essential fatty acids (EFA) and some other nonfat nutrients to organisms. The qualitative and quantitative requirements of fish and crustaceans have been extensively studied (NRC 1993, Sargent et al. 2002). Previous studies have demonstrated that dietary lipid sources may influence the growth, survival, feed efficiency, and fatty acid composition of aquatic animals including fish and shrimp, and that the qualitative and quantitative requirements of fish and crustaceans for lipids considerably differ with different species (NRC 1993, D'Abramo 1997, Sargent et al. 2002). Results suggest that many marine fish and prawns require n-3 highly unsaturated fatty acids (HUFA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to maintain normal physiology and growth. For some marine species, it is believed that DHA appears to be superior to EPA (Watanabe 1993, Mourente & Toche 1993, Xu et al. 1994, Rodriguez et al. 1997).

For mollusks, however, less is known about the nutritional value of different lipid sources. According to available information, the polyunsaturated fatty acids (PUFA) of n-3 and n-6 families are able to enhance the growth of *Haliotis discus hannai* and *H. tuberculata*. Especially, 20:5n-3 played a more prominent role in abalone nutrition (Uki et al. 1986, Mai et al. 1996a, Mai et al. 1996b). It is suggested that with different EFA composition, lipids from different sources have different nutritional values for abalone. However, no significant differences were found in the growth of *H. fulgens* fed the different oil types, olive, corn, linseed, and cod liver oils (Durazo-Beltrán et al. 2003). There is no information available about the effects of different lipid sources on the growth of *H. discus hannai*. This study was conducted to evaluate the effects of different dietary lipid sources on the growth, survival, and carcass composition of juvenile abalone *H. discus hannai*, which would provide useful information for abalone aquaculture.

MATERIALS AND METHODS

Experimental Diets and Design

Experimental basal diet was formulated to provide 28% crude protein from casein and gelatin (Table 1), which is considered to be sufficient to maintain optimum growth for *H. discus hannai* (Mai et al. 1995a). Tripalmitin (TP), soybean oil (SO), linseed oil (LO) or fish oil (FO) was individually added to the basal diet to produce four experimental diets, respectively. Dietary lipid level was maintained at 3.5%, which was considered to be an optimal level for this abalone (Mai et al. 1995b). The main fatty acid compositions of SO, LO and FO were determined by GC, and the results are given in Table 2. Procedures for diet preparation and storage were the same as those previously described by Mai et al. (1995a).

Animal Rearing

Abalone (initial weight: 0.13 ± 0.04 g; initial shell length: 10.23 ± 1.48 mm) were derived from a spawning at Mashan Fisheries Co. Shandong, China. Shell length was measured with calipers to the nearest 0.02 mm. The individual abalone was superficially dried with tissues and then weighed to 0.01 g using an electronic balance. Growth trial was conducted in a recirculation water system. Twenty-five juvenile abalone were stocked in a rearing unit, a plastic basket (20 cm \times 20 cm \times 10 cm) placed in a glass aquarium (45 cm \times 25 cm \times 35 cm), as one replicate. There were three replicates for each of the four dietary treatments.

Prior to initiation of the experiment, abalone were placed in glass aquariums and conditioned with the basal diet for 1 wk. Animals were hand-fed with the test diets at a rate equaling 5% to 10% of wet body weight once daily at 17:00. Every morning, feces and excess feed were removed to maintain water quality. The feeding trial was run for 120 days. During the 120-day experiment, water temperature was maintained at 18°C to 25°C, salinity 31 to 35, pH 7.6 to 8.0. Dissolved oxygen was not less than 6 mg/L, and there were negligible levels of free ammonia and nitrite.

*Corresponding author. Fax: +86-532-2032495; E-mail: kmai@ouc.edu.cn

TABLE 1.

Ingredient and proximate analysis of the experimental diets (g/100 g dry wt.).

Ingredients	TP	SO	LO	FO
Casein	25.00	25.00	25.00	25.00
Gelatin	6.00	6.00	6.00	6.00
Dextrin	33.00	33.00	33.00	33.00
Na alginate	20.00	20.00	20.00	20.00
Choline chloride	0.50	0.50	0.50	0.50
CM-cellulose	5.00	5.00	5.00	5.00
vitamin mix ^a	2.00	2.00	2.00	2.00
Mineral mix ^b	5.00	5.00	5.00	5.00
Tripalmitin	3.50	1.50	1.50	1.50
Soybean oil	0.00	2.00	0.00	0.00
Linseed oil	0.00	0.00	2.00	0.00
Fish oil	0.00	0.00	0.00	2.00
Proximate analysis				
Protein	28.71	28.22	28.28	28.93
Lipid	3.54	3.28	3.53	3.43
Ash	9.40	9.24	9.03	8.93

^a Vitamin mix: each 1000 g of diet contained thiamin HCl, 120 g; riboflavin, 100 mg; folic acid 30 mg; PABA, 400 g; niacin, 200 mg; Ca pantothenate, 200 mg; inositol, 4000 mg; biotin, 12 mg; vitamin E, 450 mg; menadione, 890 mg; VB₁₂, 0.18 mg; ascorbic acid, 4000 mg; retinol acetate, 100000 IU; cholecalciferol, 2000 IU; ethoxquin, 400 mg.

^b Mineral mix: each 1000 g of diet contained NaCl, 0.4 g; MgSO₄ · 7H₂O, 6.0 g; NaH₂PO₄ · 2H₂O, 10 g; KH₂PO₄, 12.8 g; Ca(H₂PO₄) · H₂O, 8 g; Fecitrate, 1.0 g; Ca-lactate 1.4 g; ZnSO₄ · 7H₂O, 141.4 mg; MnSO₄ · 4H₂O, 64 mg; CuSO₄ · 5H₂O, 12.4 mg; CoCl₂ · 6H₂O, 0.4 mg; KIO₃, 1.2 mg.

Sample Collection and Analysis

At the termination of the experiment, animals were not fed for 3 days. All abalone were collected from the cages, counted, weighed, and measured then immediately frozen (−70 °C) for subsequent analyses. Growth was expressed as weight gain rate (WGR, %) and daily increment in shell length (DISL, μm/d). The calculation formulae are as follows:

TABLE 2.

Fatty acid composition of dietary lipid sources (%area).

Fatty Acid	Lipid Source ^b		
	SO	LO	FO
14:0	0.51	0.22	ND
16:0	9.42	6.41	ND
16:1	0.24	0.16	ND
18:0	3.82	3.58	2.02
18:1n-9	21.81	21.31	3.43
18:1n-7	0.90	1.20	1.05
18:2n-6	50.60	15.49	0.82
18:3n-3	5.70	49.03	3.11
20:4n-6	ND	ND	1.09
20:5n-3	ND	ND	49.77
22:6n-3	ND	ND	21.60

ND, undetectable (<0.01 g/100 g fatty acids); SO, soybean oil; LO, linseed oil; FO, fish oil.

$$\text{WGR (\%)} = (\text{Wt} - \text{Wi}) / \text{Wi} \times 100$$

$$\text{DISL (\mu m/d)} = [(\text{SLt} - \text{SLi}) / t] \times 1000$$

Where, Wt and Wi are final and initial mean weight (g), respectively; SLt and SLi are final and initial mean shell length (mm), respectively.

The procedures for analysis of the fatty acid profiles in lipid sources and abalone carcass were modified from those described by Metcalfe et al. (1966). Briefly, fatty acid methyl esters were separated and quantified by the gas chromatograph equipment (HP5890) with a fused silica capillary column (007-CW, HP) and a flame ionization detector. The column temperature was programmed to rise from 150° up to 200° at a rate of 15° min^{−1}, from 200° to 250° at a rate of 2° min^{−1}.

Proximate analyses of the diets and abalone soft-body samples to determine protein, lipid, ash, and moisture levels were conducted using standard procedures (AOAC 1995).

Statistical Analysis

All percentage data were square-root arcsine transformed prior to analysis. Data were submitted to 1-way analysis of variance using the STATISTICA™ package. When significant differences ($P < 0.05$) were found, means were compared using the Tukey's test.

RESULTS

Survival and Growth

The data of abalone survival and growth performances are presented in Table 3. Analysis of variance showed that there was no significant difference in survival, which ranged from 90% to 96%, among the dietary treatments. However, weight gain rate (WGR) of the animals was significantly affected by the different lipid sources. Abalone fed tripalmitin (TP) diet had a significantly lower WGR (267.32%). Meanwhile, those fed the diet with EPA-enriched fish oil (FO) showed the highest WGR (413.19%). Soybean oil (SO) and linseed oil (LO) resulted in the WGRs of 382.43% and 389.04%, respectively. These later three WGR values were not significantly different from each other. The trend of daily increment in shell length (DISL) changing with different dietary lipids was similar to that of WGR. However, the effects of dietary lipids on DISL were not significant.

Fatty acid Composition in Carcass

Fatty acid composition in abalone carcass is shown in Table 4. Different dietary lipids significantly influenced the fatty acid patterns in carcass, particularly the polyunsaturated fatty acids (PUFA). Abalone fed TP accumulated a higher level of palmitic acid (16:0) in tissues. There were significantly higher levels of EPA (20:5n-3, 12.0%) and DHA (22:6n-3, 2.6%) in the abalone fed diet with FO than those in all other treatments. The highest values of linoleic acid (18:2n-6) and arachidonic acid (20:4n-6, ARA) were found to be 1.64% and 6.03%, respectively, in abalone fed the diet with SO. Abalone fed LO had the highest values of linolenic acid (18:3n-3, 2.97%), and had a significantly higher level of EPA (7.86%) than those fed the diet with TP (5.87%) or with SO (4.61%), but it was significantly lower than that resulted from feeding FO diet (12.0%). Contents of carcass EPA in all experimental treatments were generally higher than those of DHA.

Carcass Proximate Composition

Data on abalone carcass moisture, protein, and lipid contents are given in Table 5. Among the dietary treatments, there were no

TABLE 3.

Effects of the experimental diets supplemented with different lipid sources on growth, survival of juvenile abalone, *Haliotis discus hannai*.

Diets	Initial Weight (mg)	Final Weight (mg)	Weight Gain Rate (%)	Initial Shell Length (mm)	Final Shell Length (mm)	DISL ($\mu\text{m}/\text{day}$)	Survival Rate (%)
TP	132.00 \pm 8.51	508.83 \pm 12.27 ^b	267.32 \pm 7.89 ^b	10.35 \pm 0.11	17.35 \pm 0.08	58.26 \pm 1.36	92 \pm 6.93
SO	128.70 \pm 8.60	620.12 \pm 30.49 ^a	382.43 \pm 19.36 ^a	10.15 \pm 0.06	17.80 \pm 0.57	63.28 \pm 5.13	92 \pm 4.00
LO	125.33 \pm 8.20	610.53 \pm 25.86 ^a	389.04 \pm 46.82 ^a	10.20 \pm 0.06	18.02 \pm 0.36	65.47 \pm 3.21	90 \pm 9.24
FO	131.33 \pm 7.57	671.50 \pm 34.30 ^a	413.19 \pm 53.09 ^a	10.21 \pm 0.09	18.30 \pm 0.42	67.14 \pm 4.88	96 \pm 0.00
ANOVA							
F	0.41	19.01	9.22	3.00	3.04	2.86	0.43
P	0.75	0.00	0.01	0.10	0.09	0.10	0.74

DISL, daily increment in shell length.

TP, Tripalmitin; SO, soybean oil; LO, linseed oil; FO, fish oil.

^{a-b} Means in the same column sharing a common superscript letter were not significantly different ($P > 0.05$) as determined by Tukey's test.Mean \pm SD, $n = 3$.

significant differences in abalone carcass moisture ranging from 77.1% to 78.1%, and in abalone carcass protein ranging from 64.8% to 66.2%. However, the significant effect of the dietary treatments on abalone carcass lipid contents was observed. The diet containing TP resulted in significantly lower carcass lipid (4.7%) than those containing either SO (6.1%), LO (6.0%) or FO (5.6%).

DISCUSSION

Previous studies on the requirement of *H. discus hannai* for essential fatty acids using purified diets or macroalgae demonstrated that EPA (20:5n-3) played a prominent role in the nutrition of abalone, and linoleic acid together with other n-3 and n-6 PUFA also contributed to the faster growth of *H. discus hannai* (Uki et al. 1986, Mai et al. 1996a, Mai et al. 1996b). During the 120-day feeding trial of the present study, the mean body weights of abalone in all the four treatments increased by high percentages, from 267.3% to 413.1%. This means that, on one hand, the carcass storage of essential fatty acids are probably able to support abalone growth for a long period. On the other hand, abalone is probably able to biosynthesize essential fatty acids, to a certain extent, from

different lipid sources. It can be seen from Table 4 that the carcass of abalone fed the TP diet showed fairly high levels of 18:2n-6, 20:4n-6 and 20:5n-3, indicating that active elongation and desaturation of palmitic acid take place in *H. discus hannai*. Similarly, the highest level of 20:4n-6 in carcass was found in the treatment with dietary SO that contains the highest level of 18:2n-6 (50.6%), and significantly higher carcass level of 20:5n-3 was observed in abalone fed the diet with LO, which contains the highest level of 18:3n-3 (49.0%), than those fed diets with TP or SO. It is suggested that *H. discus hannai* have the ability to bio-convert 18:2n-6 to 20:4n-6, and 18:3n-3 to 20:5n-3. Similar findings have been reported in *H. laevigata* (Dunstan et al. 1996) and *H. fulgens* (Durazo-Beltrán et al. 2003).

Abalone is probably able to biosynthesize highly unsaturated fatty acids (HUFA), to a certain extent, by elongation and desaturation of short-chain fatty acids, however, the differences in weight gain rate were observed among the dietary treatments of different lipid sources (Table 3). This is probably due to the fact that the capacity of abalone to biosynthesize essential fatty acids is insufficient to support normal growth, and that biosynthesis of essential fatty acids is an energy-consuming process that could suppress the

TABLE 4.

Effects of the experimental diets supplemented with different lipid sources on fatty acid compositions in the carcass of juvenile abalone, *Haliotis discus hannai*.

Fatty Acid	TP	SO	LO	FO	ANOVA	
					F	P
14:0	2.65 \pm 0.09	2.08 \pm 0.30	2.57 \pm 0.37	2.90 \pm 0.15	1.02	0.43
16:0	18.99 \pm 1.99	15.57 \pm 1.19	15.64 \pm 0.42	15.29 \pm 2.05	3.77	0.06
16:1	1.15 \pm 0.06	0.97 \pm 0.02	1.05 \pm 0.08	1.05 \pm 0.08	0.15	0.92
18:0	5.29 \pm 1.22	5.85 \pm 0.38	5.69 \pm 0.32	5.39 \pm 0.64	0.17	0.91
18:1n-9	7.43 \pm 0.24	6.71 \pm 0.41	6.51 \pm 0.58	5.95 \pm 0.17	0.37	0.77
18:1n-7	5.55 \pm 0.41	4.48 \pm 0.28	5.34 \pm 0.52	4.36 \pm 0.37	1.74	0.23
18:2n-6	1.29 \pm 0.09 ^{ab}	1.64 \pm 0.07 ^a	0.65 \pm 0.04 ^{ab}	0.28 \pm 0.01 ^b	4.91	0.03
18:3n-3	0.49 \pm 0.02 ^b	0.39 \pm 0.02 ^b	2.97 \pm 0.49 ^a	0.58 \pm 0.03 ^b	54.95	0.00
20:4n-6	4.78 \pm 0.90 ^{ab}	6.03 \pm 0.52 ^a	4.03 \pm 0.34 ^b	3.55 \pm 0.46 ^b	9.70	0.01
20:5n-3	5.87 \pm 0.64 ^c	4.61 \pm 0.01 ^c	7.86 \pm 0.36 ^b	12.00 \pm 1.15 ^a	53.48	0.00
22:6n-3	0.89 \pm 0.02 ^b	0.93 \pm 0.03 ^b	1.28 \pm 0.13 ^b	2.59 \pm 0.10 ^a	48.19	0.00

TP, Tripalmitin; SO, soybean oil; LO, linseed oil; FO, fish oil.

^{a-c} Means in the same line sharing a common superscript letter were not significantly different ($P > 0.05$) as determined by Tukey's test.Mean \pm SD, $n = 3$.

TABLE 5.

Effects of different lipid sources on the carcass chemical composition of juvenile abalone, *Haliotis discus hannai*.

Diet	Moisture	Protein	Crude Lipid
TP	77.81 ± 0.66	65.35 ± 0.42	4.74 ± 0.11 ^c
SO	77.19 ± 0.42	64.83 ± 0.41	6.11 ± 0.25 ^a
LO	77.50 ± 1.08	65.18 ± 0.86	6.00 ± 0.18 ^{ab}
FO	77.14 ± 0.85	66.17 ± 0.32	5.56 ± 0.20 ^b
ANOVA			
F	0.731	3.270	18.551
P	0.591	0.080	0.000

TP, Tripalmitin; SO, soybean oil; LO, linseed oil; FO, fish oil.

^{a-c} Means in the same column sharing a common superscript letter were not significantly different ($P > 0.05$) as determined by Tukey test.

Mean ± SD, $n = 3$.

growth of abalone fed diets with short-chain fatty acids. The present study and previous ones have demonstrated that HUFAs are superior to short-chain PUFAs in enhancing abalone growth (Uki et al. 1986, Mai et al. 1996a, Mai et al. 1996b). Lipid sources, rich in HUFA, mainly come from marine origins, such as fish oils. However, fish oils are increasingly in short supply globally, and

intensive studies are underway on fish oil substitution in the aquaculture feed industry. Hence, further studies should be conducted to determine the optimal ratio of fish oils to plant oils in diets for abalone. Durazo-Beltrán et al. (2003) did not find significant differences in the growth of *H. fulgens* fed the different oil types (olive, corn, linseed, and cod liver oils). The authors assumed that the lack of a growth response to the different lipid sources and different levels of HUFA may be due to insufficient duration (75 days) of the experiment to achieve an essential fatty acid deficiency.

Fatty acid biosynthesis in fish and crustaceans has been extensively studied and it has been shown that the overall conversion of 18-carbon PUFA to HUFA occurs poorly in the marine species studied so far (Ito & Simpson 1996, D'Abramo 1997, Sargent et al. 2002). For abalone, however, there is still no direct evidence on the pathways of fatty acid biosynthesis. Further investigations are needed to elucidate detailed pathways of fatty acid biosynthesis and their regulation in abalone and other mollusks.

ACKNOWLEDGMENTS

This study was financially supported by grant No. 30200215 from the National Natural Science Foundation of China (NNSFC) and grant No. 39925029 from National Science Fund for Distinguished Young Scholars (NNSFC).

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DIETARY PANTOTHENIC ACID REQUIREMENT OF JUVENILE ABALONE, *HALIOTIS DISCUS HANNAI* INO

WEI ZHU, KANGSEN MAI,* BEIPING TAN, AND GETIAN WU

The Key Lab of Mariculture (Ministry of Education), Ocean University of China, Qingdao 266003, People's Republic of China

ABSTRACT Juvenile abalone, *Haliotis discus hannai* Ino, of 0.55 ± 0.02 g in body weight, and 15.48 ± 0.31 mm in shell length, were fed with six purified diets containing 0, 9.8, 19.8, 39.7, 79.4, 158.5 mg Ca-pantothenic acid/kg diet for 16 wk. The Ca-pantothenic acid was microbound with sodium alginate, and other water-soluble vitamins were encapsulated with sodium alginate, by an emulsion coacervation process, prior to supplementation in experimental diets. The dietary Ca-pantothenic acid concentration did not affect the body composition of abalone. Weight gain (WG), daily increment in shell length (DISL), and tissue pantothenic acid were significantly affected by dietary pantothenic acid. According to broken-line regression analysis, 21 mg Ca-pantothenic acid/kg diet was found to satisfy the maximum growth of abalone, whereas on the basis of the pantothenic acid concentration in the viscera and muscle of the experimental abalone, 24 and 26 mg Ca-pantothenic acid/kg diet were required, respectively. Hence, the optimum dietary Ca-pantothenic acid requirement for *H. discus hannai* Ino is estimated to be 26 mg/kg diet.

KEY WORDS: abalone, *Haliotis discus hannai* Ino, Ca-pantothenic acid, requirement, feeding and nutrition, molluscs

INTRODUCTION

Pantothenic acid (PA) has crucial roles in intermediary metabolism as a constituent of coenzyme A (CoA) and phosphopantotheine. As a part of coenzyme A, pantothenic acid transfers acyl groups in enzymatic reaction such as oxidation of pyruvate and fatty acids (NRC 1983).

Pantothenic acid deficiency signs in fish are usually growth retardation, anorexia, abnormal swimming behavior, and death (Halver 1989). The most common signs of PA deficiency are clubbed gill and fusion of gill filaments (NRC 1981, Wilson et al. 1983, Butthep et al. 1985, Masumoto et al. 1994). Dietary PA requirement for maximum growth and prevention of deficiency signs have been reported to be 10–20 mg/kg diet for salmonids (NRC 1981), 10 mg/kg of diet for channel catfish (Murai & Andrews 1979, Wilson et al. 1983) and blue tilapia (Roem et al. 1990, Soliman & Wilson 1992), and 30–50 mg/kg for common carp (Ogino 1967).

Masumoto et al. (1994) suggested that free PA tissue concentrations measured by radioimmunoassay (RIA) are a more sensitive indicator of pantothenic acid intake in rainbow trout than reduction in appetite or reduced growth.

Abalone are large algivorous marine mollusks of the genus, *Haliotis* (Gastropoda, Prosobranchia, Archaeogastropoda, Haliotidae). They are the most commercially important gastropods in aquaculture. Water-soluble vitamin requirements of the abalone, *H. discus hannai*, were recently reported to include: vitamin C, vitamin B₁, inositol, and biotin (Mai 1998, Mai et al. 2001, Wu et al. 2002, Zhu et al. 2002). The objective of this study is to investigate the effects of dietary PA on survival, growth, and tissue PA concentration and further to estimate the optimum level of dietary PA for the abalone, *H. discus hannai*.

MATERIALS AND METHODS

Experimental Diet

Formulations of the experimental diets and their proximate composition are shown in Table 1. Crude protein level of the experimental diets was 28.2%, which is considered to be sufficient to maintain optimum growth for *H. discus hannai* (Mai et al.

1995b). Soybean oil and menhaden fish oil (1:1) was used as the basal lipid source. Dietary lipid level was 3.75%, which was sufficient to support optimum growth and provide enough EFA for the abalone (Mai et al. 1995a). The vitamin (except for Ca-PA) and mineral mixture were slightly modified from those used by Uki et al. (1986). Six experimental diets were formulated from purified ingredients to provide graded levels of Ca-pantothenic acid ranging from 0–158.5 mg/kg diet.

Ca-PA was microbound with sodium alginate prior to supplementation to the experimental diet. One gram Ca-PA was mixed well with 1-g sodium alginate; appropriate water was added and mixed. The paste was lyophilized for 24 h, and then ground to a powder (passed through a sieve with 125- μ m pore size). Other water-soluble vitamins were microencapsulated with sodium alginate by an emulsion coacervation process prior to supplementation to the experimental diets. The method of microencapsulation was modified from that described by Bodmeier and Wang (1993). A portion of sodium alginate solution (2%, w/w) containing 2% (w/w) of the vitamins to be microencapsulated, was mixed with another portion of oil containing 6.74 g/L of Span 80, stirred at 400 rpm for 15 min. The emulsified solution was slowly poured into 1% (w/w) CaCl₂ solution, with continuous stirring for 1 min, and then filtered with a vacuum filter. The harvested microcapsules were washed with cyclohexane and non-water alcohol in turn, to remove oil and water, dried at room temperature, and then kept at -20° until use. The actual quantities of Ca-pantothenic acid in diets were measured by HPLC (Table 2).

Procedures for diet preparation were based on the method of Mai et al. (1995a). All the ingredients (passed through a sieve with 125 μ m pore size) were mixed thoroughly and were made to a paste by gradually adding water (about 120%, v/w). The paste was shaped into 0.5-mm thick sheets, which were cut into 1-cm² flakes. The flakes were dipped in an aqueous solution of CaCl₂ (5%, w/v) for about 1 min, and then the surplus solution was drained naturally. The diet flakes were sealed in a sample bag and stored at -20° until use.

Experimental Procedures

Abalone juveniles (*H. discus hannai*), of 0.55 ± 0.02 g average weight and 15.48 ± 0.31 mm mean shell length, used in this experiment, were from a spawning at Mashan Fisheries Co., Shan-

*Corresponding author. Fax: +86-532-2032495; E-mail: kmai@ouc.edu.cn

TABLE 1.

Ingredient and proximate composition of the basal diet (% on dry weight basis).

Ingredients	Content (g/100g Diet)
Casein, vitamin free (Sigma Chemical, St. Louis, MO, USA)	25
Gelatin (Sigma)	6
Dextrin (Shanghai Chemical Co., Shanghai, China)	34
CM-cellulose (Shanghai Chemical Co.)	5
Sodium alginate (Shanghai Chemical Co.)	20
Vitamin mix ^a , Ca-PA free	2
Mineral mix ^b	4
Choline chloride (Shanghai Chemical Co.)	0.5
SO/MFO ^c	3.5
Proximate analysis (means of triplicates)	
Protein (%)	28.5
Lipid (%)	3.80
Ash (%)	8.27

^a See Table 2.

^b Mineral mix: each 1000 g of diet contained NaCl, 0.4 g; MgSO₄·7H₂O, 6.0 g; NaH₂PO₄·2H₂O, 10 g; KH₂PO₄, 12.8 g; Ca(H₂PO₄)₂·H₂O, 8 g; Fe citrate, 1.0 g; Ca lactate, 1.4 g; ZnSO₄·7H₂O, 141.4 mg; MnSO₄·4H₂O, 64 mg; CuSO₄·5H₂O, 12.4 mg; CoCl₂·6H₂O, 0.4 mg; KIO₃, 1.2 mg.

^c Soybean oil and menhaden fish oil (1:1) with 0.001% ethoxyquin.

dong Province of China. Thirty abalone were placed into each rearing unit, a plastic cage (20 cm × 20 cm × 15 cm) with a corrugated plastic plate as a shelter. There were three replicates for each of the six dietary treatments. All the cages were distributed randomly in a rectangle pool (1 m × 1 m × 6 m). Fresh seawater, flowing through 30-μm primary sand filters, then through 10-μm secondary composite sand filters, was continuously supplied to the pool. The flow rate was about 0.5 L per min per cage. Cages were kept in dim light by screening a large drape above. Before the experiment, the abalone underwent a week conditioning period. The animals were fed with test diets once a day at 17:00 at a satiation level with a little leftover (5% to 10% of abalone wet weight per day). The plastic cages were cleaned at 8:00 o'clock every morning to remove the uneaten feed and feces during the experimental period. The temperature of the water was 15 °C to 25 °C, salinity 30‰ to 34‰, pH 7.6 to 7.9. Dissolved oxygen was not less than 7 mg/L, and there were negligible levels of free ammonia and nitrite. The Ca-pantothenic acid in the seawater was below the level of detection. The whole feeding experiment was conducted for 16 wk.

Sample Collection and Analysis

At the termination of the experiment, all abalone from each replicate, were fasted for 3 days, then removed from the cages, counted, weighed, measured, and then frozen in -70° for subsequent chemical analysis. Growth performance is expressed as weight gain (WG, %) and daily increment in shell length (DISL, μm/day). The calculation formulae are as follows:

$$\text{WG (\%)} = [(\text{Wt}-\text{Wi}) / \text{Wi}] \times 100$$

$$\text{DISL (\mu m/day)} = [(\text{SLt}-\text{SLi}) / t] \times 1000$$

where, Wt, Wi are final and initial mean weight (g), SLt, SLi are final and initial mean length (mm), respectively; and t is the feeding experiment period (days).

TABLE 2.

Composition of vitamin mix and Ca-pantothenic acid (Ca-PA) in the diet (on dry weight basis).

Vitamin Mix (Without Ca-PA) ^a		Actual Ca-PA Content in Test Diet (mg/kg Diet)	
Vitamin	1000 g Diet	Diet	Ca-PA
Thiamin HCl	120 mg	D0	0
Riboflavin	100 mg	D10	9.4 ± 0.87
Folic acid	30 mg	D20	19.8 ± 2.35
PABA	400 mg	D40	39.7 ± 3.74
Pyridoxine HCl	40 mg	D80	79.4 ± 6.88
Niacin	200 mg	D160	158.5 ± 10.73
Inositol	4000 mg		
Biotin	12 mg		
Vitamin E	450 mg		
Menadione	890 mg		
VB ₁₂	0.18 mg		
Ascorbic acid	4000 mg		
Retinol acetate	100000 IU		
Cholecalciferol	2000 IU		

^a 400 mg ethoxyquin was used as an antioxidant.

Proximate analyses to determine protein, lipid, and moisture levels in diets and abalone carcass were conducted using standard methods (AOAC 1995). Moisture content was calculated following drying in an oven at 105° for 24 h. Crude protein was estimated by the Kjeldahl method. Crude lipid was determined after ether extraction by the Soxhlet method.

Water Stability of Ca-Pantothenic Acid in Experimental Diets

The leaching measurement of dietary Ca-PA was carried out with the modified method of Zhu et al. (2002). The experimental feeds, together with a control feed supplemented with 200 mg/kg crystalline Ca-pantothenic acid, were put into packets of 100-mm screens and placed onto the bottom of experimental cages without abalone. Temperature and flow rate were 20 ± 0.8° C and about 0.5 L per min per cage that matched those of the experiment respectively. At the end of allotted times (0, 1, 2, 6, 12 h, respectively), the remaining diets were removed from the cages and freeze dried for 24 h. Then the Ca-PA concentrations were measured by HPLC.

The Ca-PA levels in abalone tissues and diets were analyzed with HPLC by a method modified from the HP operation manual (HP 1999). Weighted viscera and muscle tissues were homogenized with 3 mL of Millipore water. Homogenates were deproteinized with saturated barium hydroxide and 10% zinc sulfate. Samples were then centrifuged (6000g for 10 min.) and the supernatants were filtered through a 0.45-μm filter prior to being analyzed by HPLC with an ODS hyzersil column (4 × 250 mm). The mobile phase A was 15 mM phosphate buffer (pH 7.5) and mobile phase B was methanol. A: B = 70: 30 was used as eluant. The flow rate was 0.8 mL/min. Pantothenic acid was detected with a diode array detector (DAD) and detecting wavelength was 245 nm. The Ca-PA (from Sigma Co.) was solved in Millipore water as a standard.

Statistical Analysis

All percentage data were square-root arcsine transformed prior to analysis. Data from each treatment were subjected to one way

TABLE 3.
Retention of Ca-pantothenic acid (%) of the experimental diets immersed in sea water for different times.

Dietary Ca-PA (mg/kg Diet)	Immersion Time				
	0	1	2	6	12
0	— ^a	—	—	—	—
9.8	100	90.7 ± 3.8 ^{a2}	84.2 ± 2.1 ^a	64.4 ± 4.1 ^a	44.2 ± 3.2 ^a
19.8	100	90.3 ± 3.0 ^a	83.0 ± 3.4 ^a	63.0 ± 3.7 ^a	43.4 ± 3.4 ^a
39.7	100	89.5 ± 3.6 ^a	82.2 ± 4.2 ^a	62.2 ± 2.5 ^a	42.4 ± 3.0 ^a
79.4	100	88.2 ± 3.2 ^a	81.3 ± 3.5 ^a	61.7 ± 4.2 ^a	41.1 ± 1.9 ^a
158.5	100	87.0 ± 2.7 ^a	78.9 ± 2.6 ^a	59.4 ± 3.3 ^a	40.5 ± 2.4 ^a
200 (crystalline)	100	65.5 ± 3.9 ^b	53.5 ± 3.7 ^b	38.5 ± 2.6 ^b	28.5 ± 1.7 ^b
ANOVA					
F value		15.421	20.522	22.384	28.671
P value		0.003	0.001	0.001	0.000

^a Not detectable.

² Means in each column not sharing a common superscript are significantly different according to Tukey test ($P < 0.05$).

Mean ± SE, $n = 3$.

ANOVA. When overall differences were significant at less than 5%, Tukey HSD test was used to compare the mean values between individual treatments. Ca-PA requirements of the juvenile abalone were estimated from weight gain (WG), daily increment in shell length (DISL), PA concentrations in the viscera and muscle using broken-line analysis (Robbins et al. 1979). Statistical analyses were performed using the STATISTICA™ package.

RESULTS

Leaching

The leaching of microbound Ca-PA in the experimental diets was significantly lower than that of crystalline Ca-PA (Table 3). The retentions of microbound Ca-PA in the diets were about 80% after 2-h immersion in seawater and about 40% after 12-h. Meanwhile, the corresponding retentions of crystalline Ca-PA in the control diet were 53.5% and 28.5% respectively. There was no significant difference in water stability among the diets containing different levels of microbound Ca-PA at each sampling time.

Survival, Weight Gain and Daily Increment of Shell Length

There were no significant differences in survival of the abalone among the dietary treatments (Table 4), which ranged from 90% to 97.8%. The weight gain (WG) was significantly affected by dietary Ca-PA after the 16-wk rearing period. The WG was lowest in the group fed Ca-PA free diet and increased from 92.0% to 147.8% as the dietary PA increased from 0 to 19.8 mg/kg and leveled off when dietary PA further increased. The change of the values of DISL was similar to WG (Table 4). It increased from 45.5 to 64.0 $\mu\text{m}/\text{day}$ when dietary Ca-PA increased from 0 to 19.8 mg/kg and reached a plateau when dietary Ca-PA further increased. Based on broken line analysis, the relationship between WG and dietary PA level (X) is $Y = 150.24 - 2.787(21 - X)$, and that of DISL is $Y = 63.88 - 0.964(20 - X)$.

Carcass Composition

The results of carcass composition are shown in Table 5. There were no significant differences in soft body protein, lipid or moisture among all the treatments ($P > 0.05$) after feeding with the

TABLE 4.
Growth and survival of abalone, *Haliotis discus hannai*, fed diets containing different levels of pantothenic acid for 16 weeks.

Dietary PA (mg/kg Diet)	Initial Weight (mg)	Initial Shell Length (mm)	Final Weight (mg)	Final Shell Length (mm)	WG ¹ (%)	DISL ² ($\mu\text{m}/\text{day}$)	Survival (%)
0	543.3 ± 32.1	15.49 ± 0.37	1044.7 ± 94.9 ^{a3}	20.58 ± 0.02 ^a	92.0 ± 13.4 ^a	45.5 ± 2.74 ^a	96.7 ± 2.74
9.8	547.8 ± 10.7	15.57 ± 0.15	1202.3 ± 65.7 ^{ab}	21.48 ± 0.48 ^{ab}	119.4 ± 10.7 ^a	52.7 ± 4.24 ^{ab}	97.8 ± 3.16
19.8	596.7 ± 12.0	15.78 ± 0.39	1477.4 ± 80.9 ^b	22.95 ± 0.53 ^b	147.8 ± 19.2 ^{ab}	64.0 ± 2.79 ^b	90.0 ± 0.00
39.7	533.3 ± 14.5	15.26 ± 0.39	1317.2 ± 76.9 ^{ab}	22.17 ± 0.63 ^{ab}	146.8 ± 11.3 ^{ab}	62.3 ± 3.73 ^b	95.5 ± 5.63
79.4	555.6 ± 28.3	15.44 ± 0.23	1440.4 ± 104.4 ^b	22.72 ± 0.56 ^b	158.9 ± 9.99 ^b	65.1 ± 4.52 ^b	97.8 ± 3.16
158.5	543.3 ± 63.8	15.36 ± 0.65	1321.2 ± 78.4 ^{ab}	22.59 ± 0.86 ^b	143.97 ± 10.6 ^{ab}	64.6 ± 5.34 ^b	97.8 ± 3.16
ANOVA							
F value	1.417	0.636	7.120	4.998	10.960	8.138	1.062
P value	0.287	0.677	0.003	0.010	0.000	0.001	0.429

¹ Weight gain.

² Daily increment of shell length.

³ Means in each column not sharing a common superscript are significantly different according to Tukey's test ($p < 0.05$).

Means ± S.E., $n = 3$.

TABLE 5.

Soft body composition of juvenile abalone, *H. discus hannai* fed diets with graded levels of Ca-Pantothenic acid for 16 weeks.

Number of Diets	Dietary Ca-PA (mg/kg Diet)	Soft Body Protein (%) ¹	Soft Body Lipid (%) ^a	Soft Body Moisture (%)
D0	0	54.9 ± 4.1	7.5 ± 0.14	75.7 ± 3.4
D10	9.8	53.3 ± 3.4	7.9 ± 0.42	79.5 ± 5.7
D20	19.8	52.8 ± 1.7	7.6 ± 0.23	76.3 ± 2.8
D40	39.7	55.0 ± 2.5	7.5 ± 0.30	78.5 ± 3.6
D80	79.4	52.7 ± 4.6	8.1 ± 0.27	77.4 ± 2.9
D160	158.5	53.5 ± 3.2	7.8 ± 0.51	79.4 ± 4.6
ANOVA				
F value		0.469	1.982	0.582
P value		0.793	0.143	0.824

¹ On dry weight basis.

Means ± S.E., *n* = 3.

feeds containing graded levels of Ca-PA for 16 wk. Soft body protein, lipid, and moisture of the experimental abalone ranged from 52.7% to 55.0%, 7.5% to 8.1%, and 75.7% to 79.5%, respectively.

Tissue Pantothenic Acid Concentration

Visceral and muscle concentrations of PA are shown in Table 6. They were significantly affected by the dietary Ca-PA level. PA content in the viscera increased from 5.82 to 10.74 µg/g wet tissue when dietary PA increased from 0 to 39.7 mg/kg diet, then leveled off when dietary PA further increased. Changes in the PA contents in muscle were similar to those in the viscera, but were much lower than those in viscera, ranging from 1.25–4.71 µg/g. Both of them responded in a broken-line model to dietary Ca-PA levels, the breakpoints were 24 and 26 mg/kg respectively.

DISCUSSION

Leaching of vitamins from fish feed is a serious problem in aquaculture: immersion of feed, especially if prolonged, may lead to very high rates of vitamin depletion and to insufficient or unbalanced supplies of these micronutrients to farmed animals (Marchetti et al. 1999). In the present study, to reduce leaching of dietary water-soluble vitamins during the period before being consumed, Ca-pantothenic acid was treated with microbinding with sodium alginate, and other water soluble vitamins were microencapsulated with sodium alginate by an emulsion coacervation process before supplementation. A leaching test showed that the leaching rates for the first 2 h of dietary Ca-PA in seawater was significantly reduced from 46.5% to 21.1% by the above mentioned processes (Table 3). According to previous studies, it is reliable to determine the effect of dietary pantothenic acid on abalone using these experimental feeds (Zhu et al. 2002).

The optimum PA requirement is 26 mg/kg diet according to the

TABLE 6.

Pantothenic acid (PA) in viscera and muscle of juvenile abalone, *Haliotis discus hannai* fed diets with graded levels of PA for 16 weeks.

Dietary Number	Dietary PA (mg/kg)	Visceral PA (µg/g)	Muscle PA (µg/g)
0	0	5.82 ± 0.65 ^{a1}	1.25 ± 0.19 ^a
1	9.8	8.03 ± 0.87 ^{ab}	2.52 ± 0.20 ^b
2	19.8	9.75 ± 1.23 ^b	3.76 ± 0.21 ^c
3	39.7	10.74 ± 1.25 ^b	4.29 ± 0.28 ^{cd}
4	79.4	10.48 ± 1.29 ^b	4.71 ± 0.29 ^d
5	158.5	11.02 ± 1.10 ^b	4.53 ± 0.29 ^{cd}
ANOVA			
F value		6.859	59.65
P value		0.003	0.000

¹ Means in each column not sharing a common superscript are significantly different according to Tukey test (*p* < 0.05).

Means ± SE, *n* = 3.

results of this study, which is higher than 10–20 mg/kg for salmonids (NRC 1981), 10 mg/kg of diet for channel catfish (Murai & Andrews 1979, Wilson et al. 1983), and blue tilapia (Roem et al. 1990, Soliman & Wilson 1992) but lower than 30–50 mg/kg for common carp (Ogino 1967). Abalone are slow feeders. The extended period before consuming food increases the leaching of nutrients, including water-soluble vitamins like Ca-PA. In the present study, leaching of the vitamin still exists although the microbinding technique was used. If the water stability of dietary Ca-PA is further improved, the requirement of dietary Ca-PA for this species will be further reduced.

Masumoto et al. (1994) pointed out that free PA in the gill, heart, and liver were 15.2–81.5, 24.4–48.1, 68.6–83.7 nmol/g tissue of rainbow trout, and suggested that free PA in the gill and heart are sensitive indicators. In the present study, the free PA in the viscera and muscle of the abalone measured by HPLC were 5.82–11.02 µg/g viscera and 1.25–4.71 µg/g muscle respectively, and they were also significantly affected by dietary PA, indicating that they were all sensitive to PA status of the abalone.

In some fish, PA deficiency can result in various deficiency signs (NRC 1981, Wilson et al. 1983, Butthep et al. 1985, Masumoto et al. 1994). In this study, there was no other overt deficiency sign found except for poor growth. This is perhaps partly due to the bottom-living habit and slow growth of abalone (Chen et al. 1991, Wu et al. 2002, Zhu et al. 2002).

ACKNOWLEDGMENTS

The authors thank Mr. Hongming Ma and Ms. Wei Xu for their kind assistance in the biochemistry analysis and Mr. Shaobo Du for their assistance in the feeding experiment. This work is supported by grants from the National Science Fund for Distinguished Young Scholars (NSFC, No.39925029), and from the Excellent Young Teachers Program of MOE, P. R. China.

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GROWTH AND TISSUE BIOCHEMICAL COMPOSITION OF *HALIOTIS FULGENS* AT ELEVATED TEMPERATURES IN BAJA CALIFORNIA UNDER TWO DRIED BROWN ALGAL DIETS

G. PONCE-DÍAZ,^{1,2,*} E. SERVIERE-ZARAGOZA,¹ I. S. RACOTTA,¹
T. REYNOSO-GRANADOS,¹ A. MAZARIEGOS-VILLARREAL,¹ P. MONSALVO-SPENCER,¹
AND D. LLUCH-BELDA²

¹Centro de Investigaciones Biológicas del Noroeste (CIBNOR), Apartado Postal 128, La Paz, B.C.S. 23000, México; ²Centro Interdisciplinario de Ciencias Marinas (CICIMAR-IPN), Apartado Postal 592, La Paz, B.C.S. 23096, México.

ABSTRACT Growth, survival, and tissue biochemical composition of juvenile green abalone *Haliotis fulgens* were recorded for two temperature regimes simulating the contrasting marine conditions, one as a normal year and the other as an El Niño 1997 to 1998 event during 120 days in the laboratory. Abalones were fed *Macrocystis pyrifera* or *Eisenia arborea*. During the experiment, juveniles were sampled for biochemical analysis at the maximum temperature, which occurred at 60 days and at 120 days. Significant differences in growth of shell length and wet body weight among treatments were recorded. The highest growth ($39 \mu\text{m}\cdot\text{day}^{-1}$ and $16.0 \text{ mg}\cdot\text{day}^{-1}$) occurred in juvenile abalone within the El Niño temperature pattern and fed *M. pyrifera*. Survival rates were approximately 95% without significant differences between treatments. At maximum temperature, juveniles within the El Niño pattern had higher levels of protein in the hepatopancreas, regardless of diet. A significant interaction between temperature regimen and food was observed for glycogen in muscle, with the highest levels in juveniles fed *M. pyrifera* within the El Niño pattern. At the end of the experiment, juveniles in the normal year pattern had higher levels of lipids in the hepatopancreas. Increased levels of protein, total carbohydrates, and glycogen in muscle were present in juveniles in the El Niño pattern, regardless of diet. Juveniles fed *M. pyrifera* had higher levels of protein and lipids in the hepatopancreas and increased levels of protein, carbohydrates, and glycogen in muscle tissue, regardless of temperature regimen. The influence of diet and temperature regimen on several biochemical components closely matched their effects on growth.

KEY WORDS: biochemical composition, carbohydrates, El Niño, glycogen, green abalone, *Haliotis fulgens*

INTRODUCTION

Long-term temperature series have shown that, during El Niño oceanographic conditions, the appearance of unusually warm surface waters in the eastern tropical Pacific Ocean is one of the most prominent aspects of El Niño (ENSO) phenomenon (Philander 1990). There is an anomalous increase of sea surface temperature and a coincident drop in nutrient concentration (Jackson 1977, Zimmerman & Kremer 1984, Hernández-Carmona et al. 2001). Along the Baja California Peninsula, the maximum sea surface temperatures recorded during an El Niño was in 1997 to 1998, around 28°C (Ponce-Díaz et al. 2003b). It has been considered the most intense of the 20th century (McPhaden 1999).

The effect of temperature on the physiology of abalone includes gonadal maturation, larval development, feed consumption, ammonia excretion, oxygen consumption, growth rate, and survival (Britz et al. 1997). Thermal tolerance limits are more restrictive in larvae and recently settled individuals, but temperature affects abalone species differently. In *Haliotis fulgens* Philippi, 1845, the upper lethal limit for juveniles (1–2 cm) is 31.5°C (LT₅₀-48 h) (Leighton et al. 1981). This suggests that there was little or no direct effect of temperature during the strong El Niño event of 1997 to 1998 on survival of green abalone juveniles along the Baja California Peninsula coast, Mexico. Nevertheless, environmental fluctuations may favor the development of some diseases, as in California (USA); high temperatures may increase the mortality rate of black abalone (*H. cracherodii* Leach, 1814) affected by withering syndrome (WS) (Friedman et al. 1997). Mass mortality of black abalone was recorded after the occurrence of the 1984 El

Niño (Haaker et al. 1992). Friedman et al. (2002) found an intracellular bacterium (order Rickettsiales) as the etiologic agent for that die off.

Availability of a major food of abalone, the brown alga *Macrocystis pyrifera* (L.) C. Ag., becomes limited during El Niño events. Warm-water events lead to large-scale declines of surface canopy fronds in *Macrocystis* due to thermal stress and reduced nutrients in local nearshore areas (North 1957, North 1971, Hernández-Carmona 1987, Dayton & Tegner 1990, Ladah et al. 1999, Hernández-Carmona et al. 2001, Guzmán del Próo et al. 2003). Feed availability may be a significant factor to be considered regarding how abalone stocks may be affected during future El Niños and global warming. Along the Baja California coast, juvenile green abalone showed high variability in size, which may be an indirect effect of the 1997 to 1998 El Niño on their food supply (Guzmán del Próo et al. 2003). This study was designed to assess the survival, growth, and biochemical composition of the tissue of juvenile green abalone (*Haliotis fulgens*) under two temperature regimes that simulate the contrasting summer marine conditions during a normal year and a severe El Niño (1997–1998) while being fed the two dried brown algal diets *M. pyrifera* and *Eisenia arborea* Aresch.

MATERIAL AND METHODS

Temperature Patterns and Natural Diet

Two elevated temperature regimes (July to November), one for a normal year (18°C to 21°C) and the other El Niño 1997 to 1998 (20°C to 28°C), were simulated in tanks following the daily measurements of sea surface temperature at Bahía Asunción, Baja California Sur. The normal year data was obtained from an average

*Corresponding author. E-mail: gponce04@cibnor.mx

of 1992 to 1996, and the El Niño year from data obtained during 1997 (Fig. 1a) (Ponce-Díaz et al. 2003a). Controlled temperatures in laboratory tanks varied $\pm 2^\circ\text{C}$, which was related to the precision of the equipment used to control the seawater temperature (Fig. 1b).

In each temperature regimen, 2 brown algae, *M. pyrifera* and *E. arborea*, were used as food. *M. pyrifera* is considered a major food of abalone. Most fronds are lost during intense El Niño events (Hernández-Carmona et al. 2001). *E. arborea* is also believed to be an important species as a potential food for abalone along Baja California (Guzmán del Prío et al. 1972, Serviere-Zaragoza et al. 1998). Its populations decrease during El Niño events, but do not disappear (Hernández-Carmona et al. 2001). *M. pyrifera* and *E. arborea* (blades excluding pneumatocysts) were collected from the central coast of Baja California peninsula, Mexico. Blades were air dried and stored in cardboard boxes at 20°C to preclude deterioration. Diets were rehydrated before administration.

Experimental Procedure

Growth and survival of 312 hatchery-produced, green abalone *Haliotis fulgens* (average starting size $29.0 \text{ mm} \pm 4.3$ [sd] shell length and average starting wet body $2.37 \text{ g} \pm 1.1$ [sd] weight) were recorded for a 120 d experiment. Experimental animals were held in 16-L fiberglass rearing tanks ($50 \times 30 \times 35 \text{ cm}$, LWH). Abalone were marked with plastic tags glued to the shell. Rehydrated diets were given *ad libitum* in the afternoon at intervals of 2 days. Three replicate tanks were used for each of 4 treatment conditions: El Niño-*M. pyrifera*; El Niño-*E. arborea*; Normal-*M. pyrifera*, and Normal-*E. arborea*. Each tank contained 26 abalone

(12 tanks). Treatments were randomized among tanks. Every second morning, uneaten food and feces were removed. Micro algae growing on the inner walls of tanks were removed twice a week with a brush. Filtered ($10 \mu\text{m}$) seawater was supplied at a flow-through rate of 70 mL/min giving about 6 water exchanges/day. The water was vigorously aerated. Seawater temperatures lower than 24°C were maintained with a flow trough in a chiller. Higher temperatures were maintained with 100-W heaters immersed in each tank (VISI-THERM).

Growth

Shell length was measured with a vernier caliper, and wet body weight with an electronic balance (nearest 0.001 g) at 0, 30, 58, 97, and 120 days. Dead animals were removed and replaced to maintain the standard density, although data from these animals were not used in the analysis. Daily growth rates for shell length ($\text{GR}_{\text{SL}} = (\text{SL}_f - \text{SL}_i)/T$) and wet body weight ($\text{GR}_{\text{BW}} = (\text{BW}_f - \text{BW}_i)/T$) were calculated. SL_f = mean final shell length, SL_i = mean initial shell length, BW_f = mean final wet weight, BW_i = mean initial wet weight, and T = time in days.

Biochemical Analysis

At both temperature regimes, specimens were sacrificed for composition samples twice, once at the maximum temperature (60 days after the beginning of the experiment) and the other at the end of the study (120 days after the beginning of the experiment). On each date, fifteen animals from each treatment were sampled at random. The hepatopancreas and shell muscle were dissected, weighed (wet wt), and stored at -70°C for biochemical analysis.

Hepatopancreas was homogenized with 0.5 mL of 35% saline solution and muscle with 5 mL of 10% trichloroacetic acid (TCA) with a mechanical homogenizer (Vir-Tis). Protein was determined after digestion with 0.1 N NaOH (hepatopancreas) and 5 N NaOH (muscle) (Bradford 1976) and total carbohydrates by the anthrone method (Roe 1955). In hepatopancreas, total lipids were determined by the sulphophosphovanillin method (Barnes & Blackstock 1973). In muscle, glycogen was extracted with absolute ethanol and the anthrone method (Van Handel 1965). Levels of all fractions are reported as $\text{mg}\cdot\text{g}^{-1}$ of tissue (wet weight). The water content in hepatopancreas was $69\% \pm 4\%$ (sd) and in muscle $76\% \pm 3\%$ (sd) ($n = 50$).

STATISTICAL ANALYSES

At the beginning of the assay, statistical differences in shell length and wet body weight among replicates were not detected ($P < 0.05$). At 60 and 120 days, two-way ANOVA was performed to assess significant differences in growth variables and in biochemical composition of hepatopancreas and muscle, using temperature regimen (normal year or El Niño) and diet (*M. pyrifera* or *E. arborea*) as independent variables (Sokal & Rohlf 1995). Mean comparisons were done by Tukey test only when there was a significant effect of the interactions ($P < 0.05$). Statistical analysis was done with STATISTICA 6.0 software.

RESULTS

Growth

Shell length (SL) and wet body weight (BW) growth ($P < 0.01$) was greater in juveniles fed *M. pyrifera* than juveniles fed *E. arborea*, regardless of temperature regimen. Nevertheless, both SL and BW growth were affected by diet in relation to the temperature

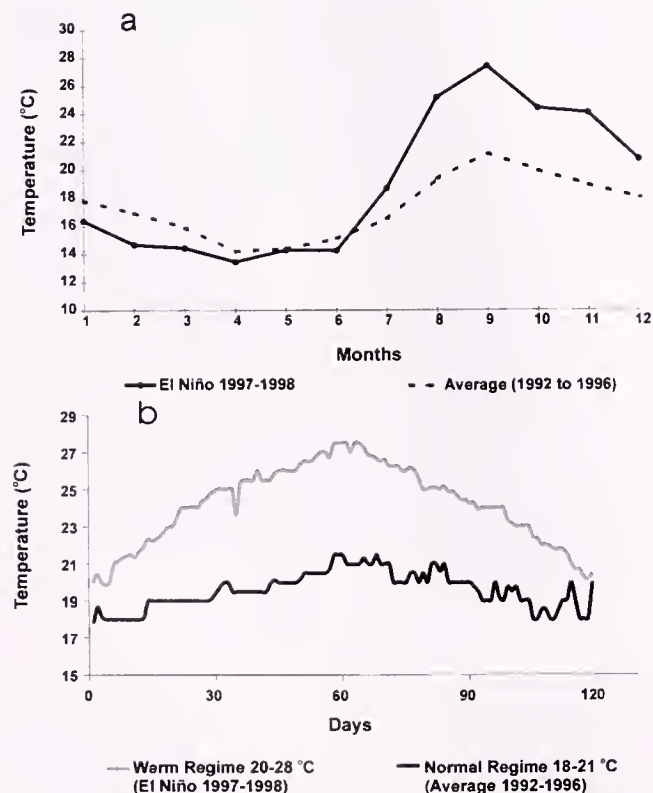


Figure 1. a. Monthly average seawater surface temperature in Bahía Asunción, measured from 1992 to 1996 and during El Niño 1997 to 1998. b. Daily average seawater temperature simulated in laboratory aquaria to represent a normal year and an El Niño year (1997 to 1998).

regimen (interaction: $P < 0.05$). At the end of the study period, the greatest mean SL and BW ($34.94 \text{ mm} \pm 0.60$, sd and $4.61 \text{ g} \pm 0.24$, sd) occurred in juveniles under El Niño-*M. pyrifera* treatment, whereas the least mean ($30.12 \text{ mm} \pm 0.91$, sd and $2.83 \text{ g} \pm 0.33$, sd) was observed in juveniles fed *E. arborea* under El Niño-*E. arborea* treatment (Fig. 2a, 2b).

Daily growth rate of SL and BW (main effect: $P < 0.01$) were higher in juveniles fed *M. pyrifera* than juveniles fed *E. arborea*, regardless of temperature regimen, although they were affected by diet in relation to the temperature regimen (interaction: $P < 0.05$). The highest daily growth rates of SL and BW ($39 \mu\text{m}\cdot\text{day}^{-1} \pm 3$ and $16 \text{ mg}\cdot\text{day}^{-1} \pm 1$) occurred in juveniles under El Niño-*M. pyrifera* treatment, and were statistically different from juveniles fed *E. arborea*; Normal-*E. arborea*, or El Niño-*E. arborea*. The lowest daily growth rates ($11 \mu\text{m}\cdot\text{day}^{-1} \pm 3$ and $4 \text{ mg}\cdot\text{day}^{-1} \pm 1$) was in juveniles fed *E. arborea* under El Niño-*E. arborea*, and were statistically different from juveniles fed *M. pyrifera*; Normal-*M. pyrifera*, and El Niño-*M. pyrifera* (Fig. 3a, 3b). The mean percent (\pm sd) of survival was $96\% \pm 4\%$ in Normal-*M. pyrifera*, $95\% \pm 5\%$ in El Niño-*M. pyrifera*, $94\% \pm 6\%$ in Normal-*E. arborea*, and $93\% \pm 2\%$ in El Niño-*E. arborea*, without significant differences between treatments (not shown).

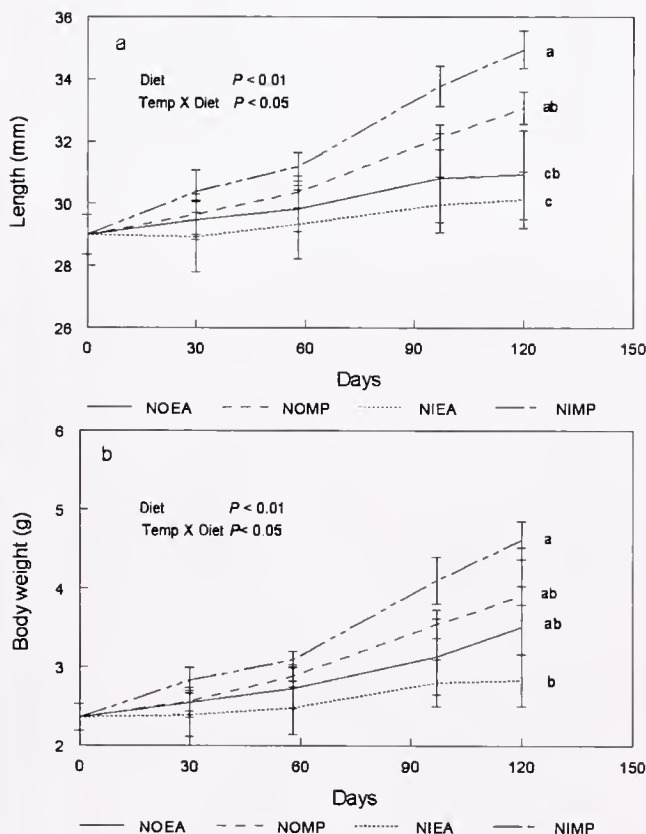


Figure 2. Mean \pm SD for triplicate groups of $n \leq 26$ of: (a) shell length and (b) body weight of juvenile green abalone *H. fulgens*. To each date, the results of bifactorial ANOVA are inserted in the figure. Factors considered for the analysis were temperature regimen (NO = normal or NI = El Niño) and diet (MP = *M. pyrifera* or EA = *E. arborea*). The main effects and interactions are shown only when significant. Mean comparisons were done by Tukey test only when there was a significant effect of the interactions. Lines not sharing the same letter are significantly different ($P < 0.05$).

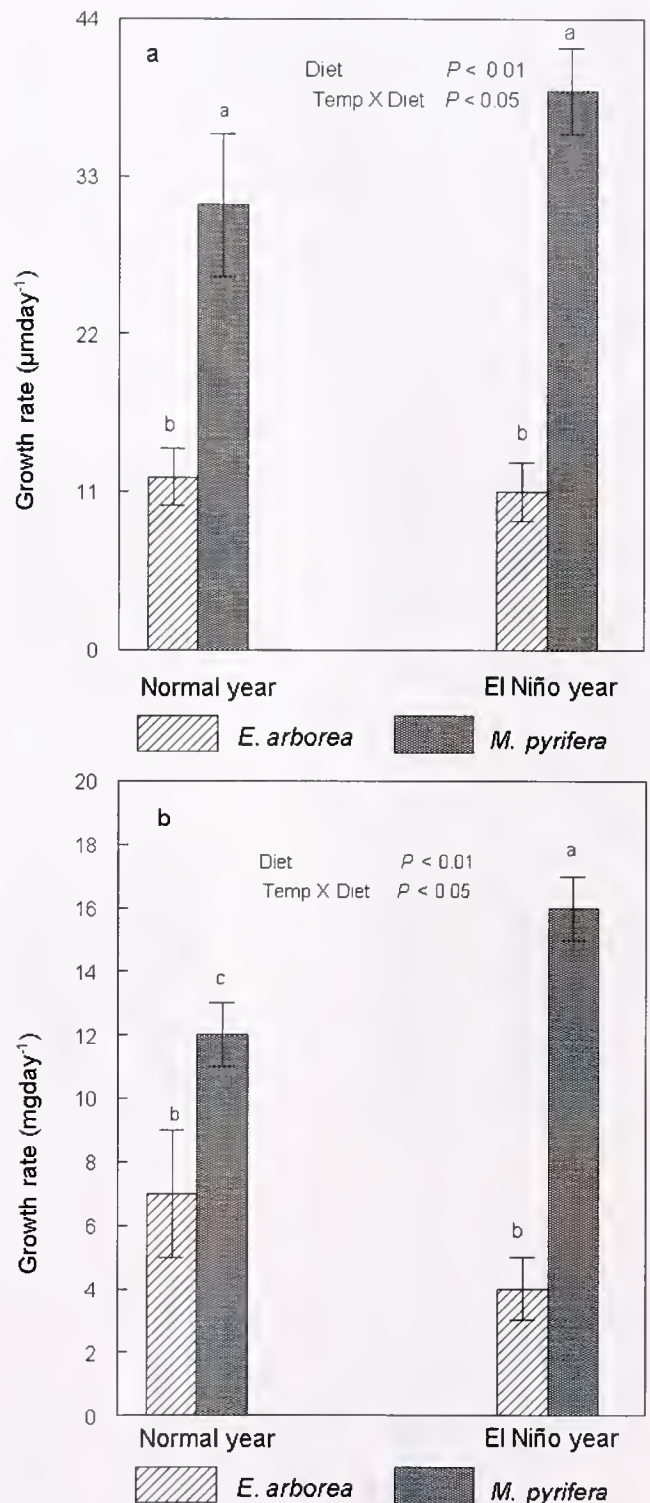


Figure 3. Mean \pm SD for triplicate groups of $n \leq 26$ for daily growth in: (a) shell length and for (b) body weight in triplicate groups of juvenile green abalone *H. fulgens*. See Figure 2 for statistical analysis.

Biochemical Variables

Hepatopancreas

At maximum temperature (60 days), the protein levels of juveniles within the El Niño pattern (global mean, 121.8 mg/g) were

higher than within the normal pattern (global mean, 99.6 mg/g) (main effect: $P < 0.01$), regardless of diet. At 120 days, juveniles fed *M. pyrifera* had higher levels of protein (global means, 107.2 mg/g vs. 88.7 mg/g) (main effect: $P < 0.05$), regardless of temperature regimen (Fig. 4a). During the assay, no significantly different effect was observed in carbohydrate levels in relation to temperature or diet (Fig. 4b). At 60 days, no significant effect in lipid levels in relation to temperature or diet was observed. At 120

days, juveniles within the Normal pattern had higher levels of lipids (global means, 9.6 mg/g vs. 6.2 mg/g) (main effect: $P < 0.01$), regardless of diet. Juveniles fed *M. pyrifera* had higher levels of lipids (global means, 10.3 mg/g vs. 5.5 mg/g) (main effect: $P < 0.01$), regardless of temperature regimen (Fig. 4c).

Muscle

At 60 days, no significant differences were observed in protein levels in relation to temperature or diet, but at 120 days, increased levels of protein (main effect: $P < 0.01$) were present in juveniles within the El Niño pattern (global means, 164.1 mg/g vs. 141.1 mg/g), regardless of diet. Juveniles fed *M. pyrifera* had higher levels of protein (158.8 mg/g vs. 146.4 mg/g) (main effect: $P < 0.05$), regardless of temperature regimen (Fig. 5a). For carbohydrates at 60 days, no significant effect was observed in relation to temperature or diet. At 120 days, increased levels of carbohydrates (main effect: $P < 0.01$) were present in juveniles within the El Niño pattern (4.6 mg/g vs. 2.9 mg/g), regardless of diet. Juveniles fed *M. pyrifera* had higher levels of carbohydrates (4.5 mg/g vs. 3.0 mg/g) (main effect: $P < 0.05$), regardless of temperature regimen (Fig. 5b). At 60 days, glycogen levels of juveniles within the El Niño pattern (1.9 mg/g) were higher than within the Normal pattern (1.3 mg/g) (main effect: $P < 0.05$), regardless of diet. A significant interaction between temperature regimen and food was observed for the carbohydrate glycogen in muscle, with the highest levels in juveniles fed *M. pyrifera* within the El Niño pattern (interaction: $P < 0.05$). At 120 days, increased glycogen (main effect: $P < 0.01$) was present in juveniles within the El Niño pattern (3.5 mg/g vs. 1.6 mg/g), regardless of diet. Juveniles fed *M. pyrifera* had more glycogen (3.3 mg/g) than those fed *E. arborea* (1.8 mg/g) (main effect: $P < 0.05$), regardless of temperature regimen (Fig. 5c).

DISCUSSION

Higher growth obtained in juveniles fed *M. pyrifera* within the two temperature regimes may be related to the relative dietary value of the common alga species along the coast of Baja California Sur. The dietary value of species, such as *E. arborea*, *Gelidium robustum* (Gardn.) Hollenb. & Abb., and the sea grass *Phyllospadix torreyi* Watson were inferior to that of the dominant algal species of southern California, *M. pyrifera* (Serviere-Zaragoza et al. 2001). In California mariculture, *M. pyrifera* is a valuable food for young red abalone, but relatively poor diet for green abalone (Leighton 1989). Growth rates for juveniles fed rehydrated *E. arborea* and *M. pyrifera* were in the range reported by other authors in animals fed fresh or rehydrated macroalgae. Growth rates of 12 and 16 $\mu\text{m}\cdot\text{day}^{-1}$ have been reported for juveniles fed fresh kelp, *M. pyrifera* (Viana et al. 1993, Viana et al. 1996), whereas values of 19 $\mu\text{m}\cdot\text{day}^{-1}$, and 46 $\mu\text{m}\cdot\text{day}^{-1}$, were obtained for juveniles fed rehydrated *E. arborea* and *M. pyrifera*, respectively (Serviere-Zaragoza et al. 2001). Difference in rates of growth among juveniles fed the same diet in different studies may be related to differences in the chemical composition of the algae used in each assay.

Biochemical composition of tissues is affected by macroalgal diets. Although most elements of the diet are nutritionally important for growth, including carbohydrate and protein components, lipid class and content may be especially vital to abalone nutrition (Nelson et al. 2002a). Foot muscle and hepatic and gonadal tissue serve as storage depots for carbohydrates and lipids, respectively (Mercer et al. 1993). In our study, juveniles fed *M. pyrifera* had

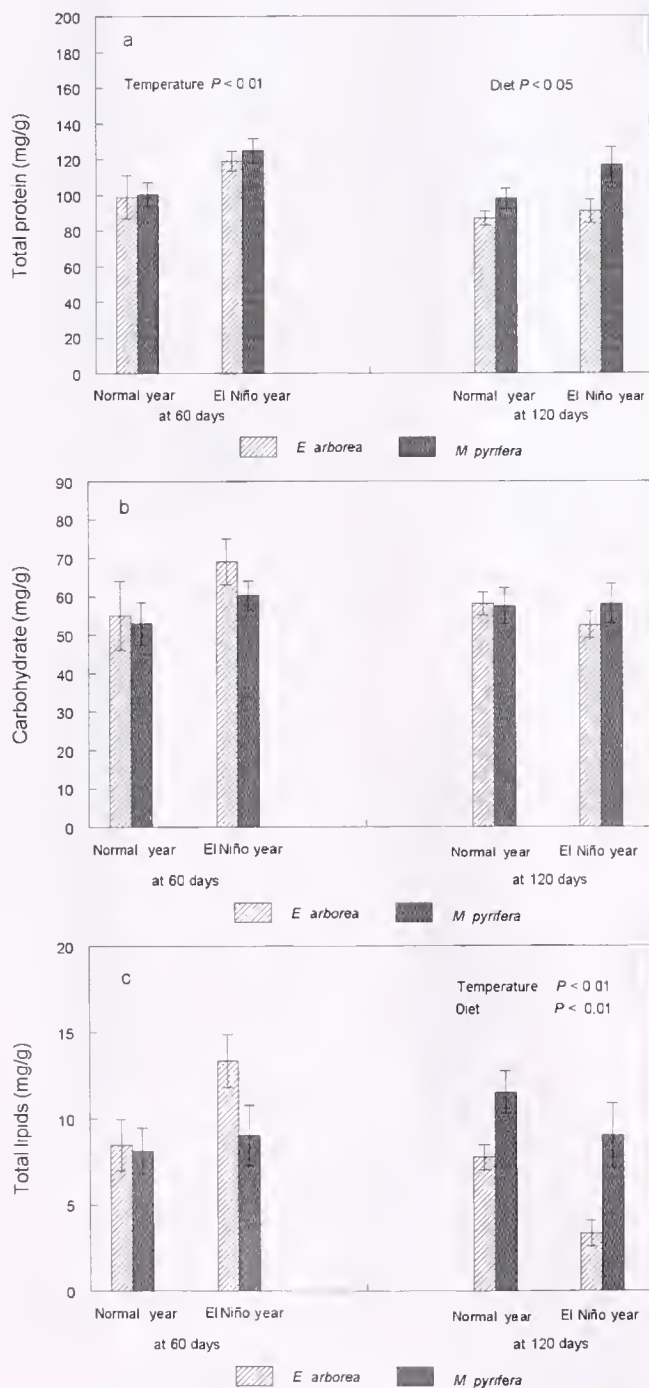


Figure 4. Mean \pm SE of hepatopancreas levels (mg·g⁻¹ hepatopancreas tissue wet weight) of total protein (a), carbohydrate (b), and total lipids (c) in juvenile green abalone *H. fulgens*. See Figure 2 for statistical analysis at 60 and 120 days.

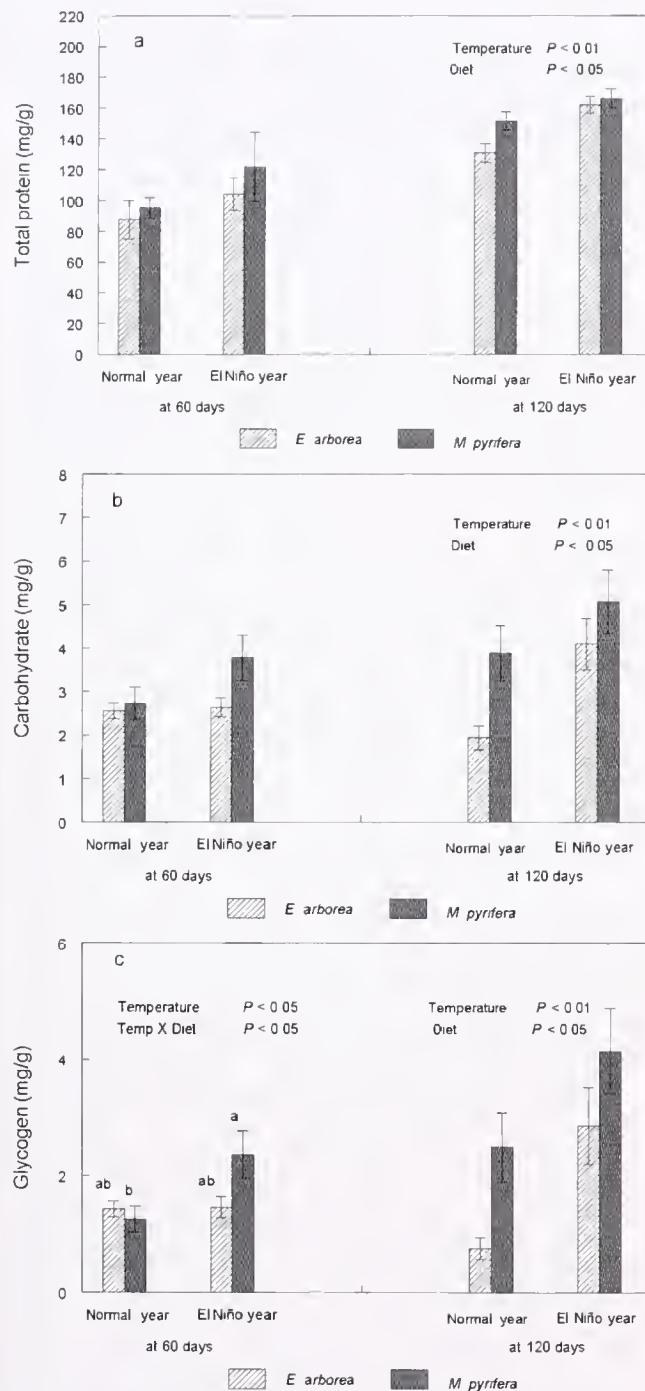


Figure 5. Mean \pm SE of muscle levels ($\text{mg}\cdot\text{g}^{-1}$ shell muscle tissue wet weight) of total protein (a), carbohydrate (b), and glycogen (c) in juvenile green abalone *H. fulgens*. See Figure 2 for statistical analysis at 60 and 120 days.

higher levels of all components in muscle than juveniles fed *E. arborea* in both temperature regimens. Muscle protein was, by far, the major component and its higher levels reflect muscle growth in accordance with the high overall growth observed in juveniles fed *M. pyrifera*. Polysaccharides stored in the foot muscle may be mainly glycogen as in many gastropods including abalone *H. discus hannai* Ito, 1953 and *H. tuberculata* Linnaeus, 1758 (Mercer et al. 1993). At the end of the assay, higher lipid levels in the

hepatopancreas of juveniles fed *M. pyrifera* were obtained, regardless of temperature. Macroalgae have lipid profiles that differ between taxa, and vary geographically and seasonally (Nelson et al. 2002b). In Baja California Sur, *E. arborea* and *M. pyrifera* have low lipid levels, ranging from 0.4% to 1.0%, without significant differences (Serviere-Zaragoza et al. 2002). Nevertheless, according to Mercer et al. (1993), lipid levels in the viscera reflect the combination of lipid levels in the diet and their bioavailability to the animals in addition to possible lipid synthesis by the abalone. This suggests differences in lipid bioavailability for these macroalgae to juvenile *H. fulgens*. Nelson et al. (2002a) found that diet and temperature influenced seasonal changes in lipid profiles, where diet most strongly affects body mass and temperature to shell length.

High survival observed in the assay ($\sim 90\%$) suggests that the maximum temperatures reached during El Niño 1997 to 1998 did not directly cause mortality in coastal juvenile green abalone populations. In culture, juvenile green abalone is tolerant of extremes in water temperature, and shows the best growth at 20°C to 28°C (Leighton 1974, Leighton et al. 1981). Low temperature reduces growth, at least in part, by affecting feeding rates and feeding duration (Uki 1981). In natural populations, high variability in size in green abalone (Guzmán del Prío et al. 2003) and reduction of growth rate in red abalone (Haaker et al. 1998) were reported during El Niños, suggesting variations in growth rate from changes in environmental conditions. This will be discussed later because it may also reflect low availability of food. In California, Friedman et al. (1997) and others suggested that high temperatures during El Niños may increase mortality of black abalone affected by the withering syndrome bacterial agent.

At the end of the experiment, hepatopancreas of juveniles within the El Niño pattern had lower levels of lipids than juveniles within the Normal pattern, regardless of diet. This suggests that during an El Niño, juveniles may use more lipid reserves than during normal years, which may in turn affect development. For macroalgae, Nelson et al. (2002b) reported that temperature induced changes in specific fatty acids, especially polyunsaturated eicosapentaenoic fatty acids, which may be important factors in gonadogenesis, and consequently, affect larval production and recruitment.

In this study, the best growth was obtained under the El Niño regimen (20°C to 28°C), providing abalone was fed *M. pyrifera*. The combined effect of high temperature and optimal food must be discussed together, because better growth was associated with higher levels of protein, carbohydrates, and glycogen in muscle. However, southern nutrient-poor water accompanies El Niño events, causing local or large-scale disappearance of the *M. pyrifera* kelp forests (North 1971, Gerard 1984, Hernández-Carmona et al. 2001). Our results suggest that higher temperatures associated with El Niño events may promote the growth of green abalone, but only if the phenomenon is not sufficiently strong to cause widespread destruction of *M. pyrifera* and other temperature-sensitive macroalgae. If El Niño events are too severe, abalone lose a main food source in Baja California Sur and must shift to other inferior algae, resulting in lower growth rates. In southern Baja California in September 1997, water temperature was high (25.3°C , anomaly $+ 5.7^{\circ}\text{C}$) and nutrients were presumably low ($< 1 \mu\text{M}$), causing large-scale disappearances of all kelp forests at the southern end of their distribution (Hernández-Carmona et al. 2001). The effects of high temperatures in the Pacific waters of the Baja California Peninsula during El Niño years have been evident

in *M. pyrifera* harvests recorded in 1958, 1983, and 1998 (Casas-Valdez et al. 2003). During El Niño 1997 to 1998, *E. arborea* populations did not disappear, but its biochemical composition changed with the increase in temperature. In *E. arborea*, tissue nitrogen content decreased from 1.95% to 0.88% between July 1997 and October 1998 monitored at Isla Asunción, in Baja California (Hernández-Carmona et al. 2001).

Although the collapse of abalone populations has been ascribed to overfishing (Prince & Guzmán del Prío 1993, Shepherd et al. 1998), impacts of El Niño events on kelp forest communities suggest that these periodic environmental influences also have contributed to these declines (Lluch-Cota et al. 1999, Guzmán del Prío et al. 2003). In this study, high temperatures (simulating El Niño) enhance growth and associated biochemical indices, if optimal food availability is maintained. Data suggest that algal species, as the main diet, may be a more important factor controlling

abalone growth than temperature or the interaction between them. The effects of climate-ocean extremes, like El Niño and La Niña conditions, should be incorporated into our understanding of changes in abalone stocks, related to each species, and its age class. Further laboratory studies will be useful to increasing our understanding of the ecologic values of these influences.

ACKNOWLEDGMENTS

The authors thank D.L. Leighton for valuable suggestions. Editorial staff at CIBNOR modified the English text. This work was supported by Sistema de Investigación del Mar de Cortés (SIMAC-Grant No. 99-0107002) and CIBNOR project PAC8. The first author was supported by a graduate fellowship from Consejo Nacional Ciencia y Tecnología (CONACyT Reg. 119827).

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UTILIZATION OF FERMENTED SKIPJACK TUNA VISCERA AS A DIETARY PROTEIN SOURCE REPLACING FISH MEAL OR SOYBEAN MEAL FOR JUVENILE ABALONE *HALIOTIS DISCUS HANNAI*

SANG-MIN LEE,^{1,*} KYOUNG-DUCK KIM² AND TAE JIN KIM³

¹Faculty of Marine Bioscience and Technology, Kangnung National University, Gangneung 210-702, Korea; ²National Fisheries Research and Development Institute, Busan 619-900, Korea ³Korean Association of Sliced Raw Fish, Busan 613-812, Korea

ABSTRACT This study was carried out to evaluate the usefulness of fermented skipjack tuna viscera (FSTV) in the diet for juvenile abalone *Haliotis discus hannai*. *Lactobacillus bulgaricus* was used for fermentation of skipjack tuna viscera. Eight isonitrogenous (about 30% crude protein) diets were formulated to include different levels (0%, 10%, 20%, and 30%) of FSTV as a replacement for either fish meal or soybean meal in diets. Three replicate groups of abalone were fed the experimental diets for 7 wk. The inclusion of FSTV up to 30% in fish meal-based diet had no significant effect on survival, body weight, shell growth, and proximate composition of abalone ($P > 0.05$). Weight gain of abalone fed the diet substituting 10% FSTV for soybean meal, was not significantly different to that of abalone fed the control diet, however this value decreased in abalone fed the 20% and 30% FSTV ($P < 0.05$). The contents of crude protein and lipid of soft body in abalone fed soybean meal-based diets were significantly affected by dietary FSTV level ($P < 0.05$). The results of this study indicate that FSTV can be used as a partial substitute protein source for fish meal or soybean meal in the formulated diet for juvenile abalone.

KEY WORDS: fermented skipjack tuna viscera, *Haliotis discus hannai*, protein source

INTRODUCTION

Abalone has a high market demand in Korea. The culture of abalone relies heavily on marine macroalgae such as *Undaria* or *Laminaria*, however, this is a problem for several reasons: unstable supply of macroalgae and increased storage cost. In addition, it has been reported that growth of abalone fed the macroalgae was lower than that of abalone fed the formulated diets (Hahn 1989, Viana et al. 1993). The better growth of abalone fed the formulated diets was attributed to the good protein quality of artificial feeds (Viana et al. 1993). In feed formulation, protein source among the dietary ingredients is very important because it is more expensive than other energy sources. Furthermore, the growth of the animal could be easily influenced by availability of dietary protein sources. Thus, high-quality proteins are necessary for a good abalone feed. Fish meal, soybean meal, and casein are the most commonly used for protein sources in abalone diets (Fleming et al. 1996). Uki et al. (1985) reported that casein was the best protein source for the diet of *Haliotis discus hannai*. But the best quality protein sources including casein are expensive. Therefore, low-cost practical alternate protein sources in diets could be of considerable economic advantage. Recently, Lee et al. (1998) suggested that the cost of *H. discus hannai* feed production could be reduced by using low cost dietary protein sources such as fish meal and soybean meal.

The utilization of waste by-products from commercial fish processing as a feed ingredient for aquaculture will be able to decrease production cost and minimize environmental pollution. Silage has proved to be a good dietary protein source for fish (Raa & Gildberg 1982). Fish silage is generally a product of high value presenting practically the similar composition as the original raw material. Previous study (Lee et al. 1998) reported that each of casein, fish meal, and soybean meal was equally good protein source for *H. discus hannai*. This study is carried out to evaluate

the utilization of fermented skipjack tuna viscera as a protein source in the diets for juvenile *H. discus hannai*.

MATERIALS AND METHODS

Preparation of Fermented Silage

The viscera of skipjack tuna (*Katsuwonus pelamis*), which was obtained from Dongwon Industry Co. (Changwon Korea), was ground and mixed thoroughly with sugar beet molasses (100 g/kg) and *Lactobacillus bulgaricus* KCTC 3188 (50 g/kg) as starter and then fermented at 35°C for 10 days in a sealed 50-L plastic bucket. After 10 days, the fermented sample was filtered by using gauzes and used for the dietary ingredient.

Experimental Diets

Two sets of experimental diets were formulated using fermented skipjack tuna viscera (FSTV) at 0%, 10%, 20%, and 30% levels replacing mainly fish meal in experiment 1 (Table 1) and soybean meal in experiment 2 (Table 2), respectively. Wheat flour and α -potato starch were used as carbohydrate, and squid liver oil was used as lipid source. Procedures for feed preparation were adapted from the method of Mai et al. (1995a). Experimental diets were dried at room temperature and stored at -25°C until used. All diets were similar in contents of crude protein (28.4% to 30.0%) and lipid (4.3% to 6.8%), which are considered to be sufficient to maintain optimum growth for *H. discus hannai* (Mai et al. 1995a, Mai et al. 1995b).

Experimental Design

Two feeding trials with completely randomized block designs were conducted to evaluate the usefulness of FSTV in the diet for juvenile *H. discus hannai* in flow-out system. Juvenile abalone were acclimated to the experimental condition for 2 wk while

*Corresponding author. E-mail address: smlee@kangnung.ac.kr

TABLE 1.

Ingredient and nutrient contents (% DM basis) of the experimental diets replacing white fish meal with silage (experiment 1).

	Silage Level (%)			
	0	10	20	30
Ingredients				
White fish meal ¹	35.0	29.0	23.0	17.0
Wheat flour	24.0	21.2	18.4	15.6
Fermented skipjack tuna viscera	—	10.0	20.0	30.0
<i>Undaria</i> powder	5.0	5.0	5.0	5.0
α -potato starch	5.0	5.0	5.0	5.0
Squid liver oil ²	4.0	2.8	1.6	0.4
Vitamin premix ³	2.5	2.5	2.5	2.5
Mineral premix ⁴	4.0	4.0	4.0	4.0
Sodium alginate	20.0	20.0	20.0	20.0
Choline salt	0.5	0.5	0.5	0.5
Nutrient contents				
Crude protein	28.4	28.7	29.1	29.4
Crude lipid	6.8	6.0	5.2	4.4
NFE ⁵	48.2	48.9	49.4	50.0
Crude fiber	1.5	1.5	1.5	1.5
Ash	15.1	14.8	14.8	14.6
n-3HUFA ⁶	1.6	1.5	1.3	1.2

¹ Imported from Russia, contained 0.015% ethoxyquin.

² Provided by E-wha Oil & Fat Ind. Co., Busan, Korea.

³ Vitamin mix contained the following amount which were diluted in cellulose (g/kg mix): L-ascorbic acid, 200; DL- α -tocopheryl acetate, 20; thiamin hydrochloride, 5; riboflavin, 8; pyridoxin hydrochloride, 2; niacin, 40; Ca-D-pantothenate, 12; myo-inositol, 200; D-biotin, 0.4; folic acid, 1.5; p-aminobenzoic acid, 20; menadione, 4; retinyl acetate, 1.5; cholecalciferol, 0.003; cyanocobalamin, 0.003.

⁴ Mineral mix contained the following ingredients (g/kg mix): NaCl, 10; MgSO₄ · 7H₂O, 150; NaH₂PO₄ · 2H₂O, 250; KH₂PO₄, 320; CaH₃(PO₄)₂ · H₂O, 200; Ferric citrate, 25; ZnSO₄ · 7H₂O, 4; Ca-lactate, 38.5; CuCl, 0.3; AlCl₃ · 6H₂O, 0.15; KIO₃, 0.03; Na₂SeO₃, 0.01; MnSO₄ · H₂O, 2; CoCl₂ · 6H₂O, 0.1.

⁵ N-free extract calculated by difference: 100 – (crude protein + crude lipid + crude fiber + ash).

⁶ Highly unsaturated fatty acids (C \geq 20).

being fed a commercial abalone diet containing 30% protein and 5% lipid. They were randomly stocked into twenty-four 20-L tanks at a density of 100 abalone in each tank. Three replicate groups of abalone (mean weight 848 mg and 821 mg in experimental 1 and 2, respectively) were fed *ad libitum* at intervals of 2 days for 7 wk. Before feeding, uneaten diets in each tank were cleaned by siphoning off. Filtrated seawater was supplied at a flow rate of approximately 3 L/min in each tank. Photoperiod was left at the natural condition, and mean temperature was 22 \pm 0.9°C during the feeding trial. Abalone in each tank were collectively weighed on the days of initiation and termination at the feeding trial after being fasted for 24 h. Two-hundred abalone samples at the beginning and all abalone at the end of feeding trial were sacrificed and stored at –70°C for chemical analysis.

Chemical Analysis

Crude protein content was determined by Kjeldahl method using Auto Kjeldahl System (Buchi B-324/435/412, Switzerland).

TABLE 2.

Ingredient and nutrient contents (% DM basis) of the experimental diets replacing soybean meal with silage (experiment 2).

	Silage Level (%)			
	0	10	20	30
Ingredients				
White fish meal ¹	5.0	5.0	5.0	5.0
Soybean meal ²	45.0	35.0	25.0	15.0
Wheat flour	12.9	14.3	15.8	17.2
Fermented skipjack tuna viscera	—	10.0	20.0	30.0
<i>Undaria</i> powder	5.0	5.0	5.0	5.0
Squid liver oil ¹	5.1	3.7	2.2	0.8
Vitamin premix ¹	2.5	2.5	2.5	2.5
Mineral premix ¹	4.0	4.0	4.0	4.0
Sodium alginate	20.0	20.0	20.0	20.0
Choline salt ¹	0.5	0.5	0.5	0.5
Nutrient contents				
Crude protein	30.0	29.7	29.5	29.2
Crude lipid	6.4	5.7	4.9	4.3
NFE ¹	48.4	49.8	51.2	52.4
Crude fiber	3.6	3.2	2.7	2.2
Ash	11.5	11.6	11.7	12.0
n-3HUFA ¹	1.2	1.2	1.1	1.0

¹ Refer to Table 1.

² Dehulled, solvent extracted.

crude lipid content by ether-extraction method, moisture content by a dry oven (105°C for 12 h), crude fiber content by an automatic analyzer (Fibertec, Tecator, Hoganas, Sweden), and ash content by a furnace muffler (550°C for 4 h). Amino acids content was determined using automatic amino acid analyzer (Hitachi 835, Japan). Tryptophan was determined according to the method of Hugli and Moore (1972). Volatile basic nitrogen was measured using Conway's microdiffusion method (Conway 1950). Lipid for fatty acids analyses was extracted by mixture of chloroform and methanol (2:1, v/v) according to the method of Folch et al. (1957). Fatty acid methyl esters were prepared by transesterification with 14% BF₃·MeOH (Sigma, St. Louis, USA), and were analyzed by using a gas chromatography (HP 5890, Hewlett-Packard, USA) with flame ionization detector, equipped with HP20M capillary column (0.25 μ m \times 30 m). Injector and detector temperatures were 250°C and 270°C, respectively. The column temperature was programmed from 150°C to 230°C at a rate of 2°C/min. Helium was used as the carrier gas. Fatty acids were identified by comparison with known standards.

TABLE 3.

Proximate analysis and volatile basic nitrogen (VBN) content of raw and fermented skipjack tuna viscera.

	Raw Viscera	Fermented Silage
Moisture (%)	73.6	69.7
Crude protein (%)	18.0	14.8
Crude lipid (%)	4.7	2.6
Carbohydrate (%)	0.7	9.7
Ash (%)	3.0	3.2
VBN (mg/100 g)	46.7	86.7

TABLE 4.

Total and free amino acids contents (mg/100 g) of raw and fermented skipjack tuna viscera.

Amino Acids	Total Amino Acids		Free Amino Acids	
	Raw Viscera	Fermented silage	Raw Viscera	Fermented Silage
Ala	1008	1070	262	640
Arg	779	665	194	507
Asp	1403	1265	39	1130
Cys	108	130	32	69
Glu	2165	1958	649	1494
Gly	982	1124	275	834
His	366	306	102	224
Ile	886	832	265	821
Leu	1466	1437	366	1433
Lys	301	247	167	241
Met	431	265	116	228
Phe	700	635	189	606
Pro	715	714	214	540
Ser	537	583	145	459
Tau	482	351	139	259
Trp	196	194	48	182
Thr	680	855	197	822
Tyr	289	158	69	126
Val	892	872	249	837
Total	14386	13661	3717	11452

Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA) using the SPSS program version 7.5 (SPSS Inc., Michigan Avenue, Chicago, Illinois, USA). Significant differences ($P < 0.05$) among mean were determined by Duncan's multiple range test (Duncan 1955).

RESULTS AND DISCUSSION

Proximate composition and volatile basic nitrogen content of the raw skipjack tuna viscera (RSTV) and fermented skipjack tuna viscera (FSTV) are shown in Table 3. The FSTV resulted in increase of carbohydrate and volatile basic nitrogen, and decrease in the contents of protein and lipid. Increase of carbohydrate in the FSTV probably originated from molasses used. The total amino acids composition (Table 4) of FSTV was similar to that of RSTV. The contents of Glu, Leu, Asp, Gly, and Ala were high among amino acids in both RSTV and FSTV. The contents of free amino acid in the FSTV were higher than those of RSTV. Considerably increased free amino acids content in the FSTV may be the result from that of the protein of RSTV, which is broken down into smaller units during storage for fermenting. Fatty acid compositions of the RSTV and FSTV are shown in Table 5, 16:0, 18:1n-9, and 22:6n-3 were most abundant fatty acids in RSTV and FSTV. The polyenes in FSTV were higher than those of RSTV. Considering compositions of amino acid and fatty acid (Mai et al. 1995b, Mai et al. 1996) in the FSTV, this fermented silage has a good nutritional condition for dietary ingredient of *H. discus hanmai*.

Growth performance of *H. discus hanmai* fed the diets replacing fish meal with different FSTV level is presented in Table 6 (experiment 1). In the present study, the inclusion of FSTV up to 30%

TABLE 5.

Fatty acid compositions (% of total fatty acids) of raw and fermented skipjack tuna viscera.

Fatty Acid	Raw Viscera	Fermented Silage
14:0	1.6	2.9
15:0	0.8	0.9
16:0	30.1	23.9
16:1n-7	4.2	7.5
18:0	10.1	7.1
18:1n-9	18.3	14.7
18:2n-6	0.3	3.9
18:3n-3	0.3	1.0
20:0	0.0	0.2
20:1n-9	0.6	0.6
20:2n-6	0.0	2.0
20:4n-6	2.9	0.6
20:5n-3	6.6	9.4
22:2n-6	0.0	1.7
22:3n-6	0.0	0.6
22:6n-3	24.2	22.1
24:1n-9	0.0	0.9
Saturates	42.6	35.0
Monoenes	23.1	23.7
Polyenes	34.3	41.3

of the fish meal-based diet had no significant effect on survival, body weight, and shell growth of abalone ($P > 0.05$). When *H. discus hanmai* fed the diets replacing soybean meal with different FSTV level (Table 6, experiment 2), weight gain of abalone fed the diet containing 10% FSTV was not significantly different to that of abalone fed the control diet, however this value decreased in abalone fed the 20% and 30% FSTV ($P < 0.05$).

The feeding value of the dietary protein source depends not only on the quantity of nutrient such as protein and essential amino acids, but also on the ability of the fish to use that nutrient (National Research Council 1993). Additionally, various characteristics of the feedstuff including nutritional values, cost, stable supply, and palatability should be considered to commercially use as the protein source in practical diet. Viana et al. (1993) reported that fish meal was a good dietary protein source for the growth of *H. fulgens*. The development of commercial feeds for aquaculture has been traditionally based on fish meal as the main protein source due to its high protein content and balanced essential amino acid profile. However, because of high price of good quality proteins including fish meal, many studies have been focused on alternative practical protein sources which have economic advantages for aquaculture (Viana et al. 1996, Lee et al. 1998, Mukhopadhyay & Ray 1999, Giri et al. 2000, Middleton et al. 2001). Fish silage has a good potential as a protein source for some fish feed (Hardy et al. 1983, Espe et al. 1992, Fagbenro et al. 1994, Fagbenro & Jauncey 1995). The results obtained from this study suggest that *H. discus hanmai* maybe use the protein from FSTV and fish meal with similar efficiency. This is agreement with other studies (Fagbenro 1994, Vidotti et al. 2002). Fagbenro et al. (1994) reported that up to 75% of fish meal protein was successfully replaced by blended lactic acid fermented fish silage:soybean meal (1:1) incorporated in diets fed to Nile tilapia fingerlings.

Weight gains (406–575 mg/abalone) of *H. discus hanmai* fed the soybean meal-based diets (experiment 2) were higher than those (279–323 mg/abalone) of abalone fed the fish meal-based

TABLE 6.

Growth performance of juvenile abalone fed the diets replacing fish meal (experiment 1) and soybean meal (experiment 2) with different silage level for 7 weeks.

	Dietary Silage Level (%)			
	0	10	20	30
Experiment 1				
Initial mean wet wt. (mg)	890 ± 39.8	812 ± 26.3	854 ± 34.2	834 ± 19.6
Final mean wet wt. (mg)	1129 ± 63.2	1097 ± 47.9	1134 ± 15.0	1156 ± 39.2
Weight gain (mg/abalone)	310 ± 35.1	284 ± 24.1	279 ± 46.9	323 ± 27.0
Survival (%)	97 ± 1.7	98 ± 1.1	98 ± 1.3	100 ± 0.0
Final soft body wet wt. (mg)	663 ± 32.0	633 ± 38.4	643 ± 21.8	640 ± 25.1
Final shell length (mm)	20.6 ± 0.24	20.1 ± 0.31	20.3 ± 0.07	20.5 ± 0.14
Final shell width (mm)	14.8 ± 0.25	14.5 ± 0.23	14.3 ± 0.09	14.7 ± 0.05
Final shell height (mm)	4.95 ± 0.058	4.92 ± 0.180	4.77 ± 0.056	4.93 ± 0.104
Soft body wt./whole body wt.	0.63 ± 0.003	0.64 ± 0.010	0.62 ± 0.006	0.60 ± 0.014
Experiment 2				
Initial mean wet wt. (mg)	848 ± 34.5	826 ± 37.3	814 ± 32.4	794 ± 17.9
Final mean wet wt. (mg)	1423 ± 52.1 ^c	1362 ± 18.6 ^{bc}	1251 ± 66.7 ^{ab}	1200 ± 23.6 ^a
Weight gain (mg/abalone)	575 ± 18.2 ^c	536 ± 43.3 ^{bc}	437 ± 44.7 ^{ab}	406 ± 6.50 ^a
Survival (%)	97 ± 0.7	99 ± 0.7	99 ± 0.7	98 ± 1.1
Final soft body wet wt. (mg)	813 ± 37.1	763 ± 52.0	720 ± 72.1	697 ± 13.3
Final shell length (mm)	21.8 ± 0.22	21.3 ± 0.38	21.2 ± 0.28	20.8 ± 0.11
Final shell width (mm)	15.7 ± 0.20	15.3 ± 0.24	15.3 ± 0.20	14.9 ± 0.16
Soft body wt./whole body wt.	0.64 ± 0.005	0.63 ± 0.012	0.63 ± 0.016	0.63 ± 0.003

Values (mean ± S.E. of three replications) in the same row not sharing a common superscript are significantly different ($P < 0.05$).

diets (experiment 1), regardless of different FSTV levels. This result suggests that soybean meal is very good protein source in diet for growth of *H. discus hannai*. However, Uki et al. (1985) reported that growth of *H. discus hannai* fed the casein diet was higher than that of abalone fed the diet containing fish meal or soybean meal. On the other hand, Lee et al. (1998) reported that each of casein, fish meal, and soybean meal was equally good protein source for *H. discus hannai*. The different growth responses to dietary protein sources among studies are probably due to feeding and abalone conditions, such as water temperature, abalone size, and dietary composition used in their studies.

There were no significant differences ($P > 0.05$) in moisture, protein, lipid, and ash contents of soft body in *H. discus hannai* fed

the diets replacing fish meal with different FSTV level (Table 7, experiment 1). When *H. discus hannai* fed the diets replacing soybean meal with different FSTV level (Table 7, experiment 2), crude protein content of soft body was higher in abalone fed control diet, while crude lipid content in abalone fed the diet containing 30% FSTV was lower than that of in the others ($P < 0.05$).

The results obtained from this study indicate that FSTV has a good potential as a substitute protein source, especially for fish meal in the formulated diet for juvenile *H. discus hannai*. The use of FSTV as an ingredient in diet for *H. discus hannai* could result in cheaper artificial feeds, making its culture more economically viable, and use of tuna viscera is potentially a profitable solution to a waste problem of the canned tuna industries.

TABLE 7.

Proximate composition (% wet basis) of the soft whole body in abalone fed the diets replacing fish meal (experiment 1) and soybean meal (experiment 2) with different silage level for 7 weeks.

	Dietary Silage Level (%)			
	0	10	20	30
Experiment 1				
Moisture	75.8 ± 0.49	75.8 ± 0.65	75.9 ± 0.46	75.5 ± 1.25
Crude protein	16.0 ± 0.25	15.9 ± 0.62	16.2 ± 0.72	16.0 ± 0.75
Crude lipid	1.41 ± 0.052	1.53 ± 0.081	1.56 ± 0.049	1.44 ± 0.086
Ash	2.44 ± 0.032	2.35 ± 0.026	2.37 ± 0.057	2.45 ± 0.133
Experiment 2				
Moisture	75.7 ± 0.83	76.0 ± 0.06	74.6 ± 0.28	76.0 ± 0.06
Crude protein	17.2 ± 0.27 ^b	16.0 ± 0.50 ^a	15.3 ± 0.22 ^a	15.6 ± 0.20 ^a
Crude lipid	1.72 ± 0.068 ^{ab}	2.01 ± 0.037 ^c	1.91 ± 0.043 ^{bc}	1.64 ± 0.100 ^a
Ash	2.37 ± 0.052	2.38 ± 0.033	2.34 ± 0.003	2.42 ± 0.065

Values (mean ± S.E. of three replications) in the same row not sharing a common superscript are significantly different ($P < 0.05$).

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EFFECTS OF VITAMINS A AND D ON SHELL BIOMINERALIZATION OF ABALONE *HALIOTIS DISCUS HANNAI*, INO

WENBING ZHANG, KANGSEN MAI,* WEI XU, QINGHUI AI, BEIPING TAN,
ZHIGUO LIUFU, HONGMING MA

The Key Laboratory of Mariculture (Ministry of Education), Ocean University of China,
Qingdao 266003, People's Republic of China

ABSTRACT A 152-day feeding trial was conducted in a recirculating water system to investigate the effects of vitamins A (retinol) and D (cholecalciferol) on shell biomineralization of the abalone *Haliotis discus hannai* Ino. Triplicate groups of juvenile abalone (initial weight: 0.35 ± 0.03 g; initial shell length: 11.31 ± 0.25 mm) were fed to satiation daily with one of the 16 semipurified diets containing 0, 1×10^3 , 1×10^5 , 1×10^6 IU/kg vitamin A and 0, 500, 1×10^3 , 5×10^3 IU/kg vitamin D in a 4×4 factorial design. The results showed that vitamin D significantly increased the ratio of calcite/aragonite in the shell, in the case of deficient (0 IU/kg) or excessive (1×10^6 IU/kg) dietary vitamin A supplementation. When dietary vitamin A supplementation ranged from 1×10^3 to 1×10^5 IU/kg, vitamin D significantly decreased the ratio of calcite/aragonite in the shell. The concentrations of Mg, Zn, and Sr in the shell were independent of the supplementation of these two vitamins. Meanwhile, the contents of Ca, P, and Cu in abalone shell increased with supplementation of the vitamins A and D, and significant interaction between vitamin A and D was observed on the concentrations of both Ca and Cu in shell. Vitamin A, instead of vitamin D, significantly increased Fe concentration in shell. Trends of the ratio of acidic/basic amino acids [(Asx + Glx)/(Lys + Arg + His)] in the shell soluble matrix protein (SMP) changing with dietary vitamin A and D were in agreement with those of the ratio of calcite/aragonite in the shell. The two vitamins did not change the shell ultrastructure as shown by scanning electron microscopy (SEM) and components of SMP expressed by SDS-PAGE.

KEY WORDS: *Haliotis discus hannai*, vitamin A, vitamin D, shell, biomineralization

INTRODUCTION

Vitamin A (retinol) is sequentially metabolized to retinaldehyde, retinoic acid for its functions in animals (Hofman & Eichele 1994, Saari 1994). All-*trans*-retinoic acid (ATRA) acts through a series of receptors, termed retinoic acid receptors (RAR) α , β , and γ . Meanwhile, 9-*cis*-retinoic acid (9-*cis*-RA) can bind another series of retinoid receptors, termed retinoid X receptors (RXR), and initiate transcription of RXR responsive genes in a ligand-dependent fashion (Rohde et al. 1999). Vitamin A is functionally related to vision, reproduction, and specific immune responses (Sporn & Roberts 1984, Dhur et al. 1991, Thompson et al. 1994). Furthermore, it induces the expression of genes associated with the terminal mineralization phase of chondrocyte maturation and promotes apatite deposition in the extracellular matrix (Iwamoto et al. 1993).

Vitamin D (cholecalciferol) requires two hydroxylations to be converted into its active hormonal form, $1\alpha,25$ -dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$]. It is activated by hydroxylation first in the liver to form 25-hydroxyvitamin D_3 [$25(OH)D_3$], and then in the kidney to form $1\alpha,25(OH)_2D_3$ (Henry & Norman 1984, Norman & Henry 1993). $1\alpha,25(OH)_2D_3$ exerts its functions through 2 pathways: nongenomic pathway (reviewed in Revelli et al. 1998) and genomic pathway (reviewed in Jones et al. 1998). The genomic pathway requires binding of $1\alpha,25(OH)_2D_3$ to the nuclear vitamin D receptor (VDR), heterodimerization with a retinoid X receptor (RXR), and interaction with specific vitamin D-responsive element (VDRE) consensus sequences of target genes (Colnot et al. 1995, Haussler et al., 1998). Vitamin D plays an important role in calcium and phosphate homeostasis (DeLuca & Schnoes 1976). Recent studies have shown that vitamin D regulates the gene expression of proteins involved in the formation of mineralized tissues. $1\alpha,25(OH)_2D_3$ stimulates the expression of osteocalcin, which is a

small extracellular bone matrix protein with a capacity for specific Gla (γ -carboxyglutamic acid)-dependent binding of Ca^{2+} (Mahonen et al. 1990). Such function of $1\alpha,25(OH)_2D_3$ was further demonstrated by later studies (Luegmayer et al. 1998). In addition, $1\alpha,25(OH)_2D_3$ was found to be able to regulate the expression of a tooth-specific gene: amelogenin gene (Papagerakis et al. 1999). Amelogenins are the major (90%) matrix proteins for forming enamel (Sasaki & Shimokawa, 1995). Amelogenesis is a multi-stage process that generates a fully mineralized layer of enamel on the crowns of teeth (Smith & Nanci 1995). This process is controlled by ameloblasts, which secrete an extracellular protein matrix and provide a gel scaffold to support apatitic crystal growth (Fincham & Simmer 1997).

In contrast to bone and tooth, mollusc shell is mostly composed of calcium carbonate ($CaCO_3$) (95% to 99% in weight), which is usually in the crystallographic form of calcite or (and) aragonite (Currey 1999). The shell contains a small amount of protein and polysaccharide (1% to 5% in weight). The organic material is responsible for the fracture toughness of the shell, which is 3000 times greater than pure crystalline aragonite (Weiner 1986). The organic matrix is also involved in the crystallization process: some parts may enhance, others may inhibit the crystal formation of calcium carbonate (Belcher et al. 1996, Weiss et al. 2000). It has been demonstrated that the soluble matrix proteins (SMP) play an important role in the control of crystal polymorphism phase switching and orientation (Belcher et al. 1996, Falini et al. 1996).

Shell biomineralization can be influenced by factors such as environmental (Kennedy et al. 1969, Almeida et al. 1998) and also the metabolic state of the organism (Wilkes & Crenshaw 1979). It was demonstrated in our previous studies that dietary Zn and P changed the character of mineralogy and the pattern of SMP in the shell of the abalone *Haliotis discus hannai* Ino (Zhang et al. 2002, Mai et al. 2003). Either vitamin A or D plays an important role in mineralization of bone and tooth as described earlier. There is no information available on the effects of vitamin A and D on mollusc shell biomineralization. There is potential for interaction between

*Corresponding author. Fax: +86-532-2032495; E-mail: kmai@ouc.edu.cn

vitamin A and D signaling pathways, because RXR is needed for the two vitamins to exert their functions (Glass 1994, Carlberg 1995). Furthermore, such interaction influences bone mineralization in the rat (Rohde et al. 1999) and the performances of growth and metabolism in *H. discus hanai* (Zhang et al., unpublished data). The aim of this study is to investigate whether vitamin A and D have effects on shell biomineralization in *H. discus hanai*.

MATERIALS AND METHODS

Animals, Diets and Experimental Treatments

Abalone juveniles (initial weight: 0.35 ± 0.03 g; initial shell length: 11.31 ± 0.25 mm) used in this experiment were derived from a spawning at Huaxin Fisheries Co., Shandong, China.

Diets were formulated with purified ingredients. The composition and the proximate analysis of the diets are presented in Table 1. The basal diet contained 32.8 IU vitamin A/kg diet and 25.6 IU vitamin D/kg diet, analyzed by high-performance liquid chromatography (HPLC). Experimental diets with 4 levels of vitamin A (0 , 1×10^3 , 1×10^5 and 1×10^6 IU/kg) and 4 levels of vitamin D (0 , 500, 1×10^3 and 5×10^3 IU/kg) were prepared by adding appropriate quantities of all-*trans*-retinol acetate and cholecalciferol to the basal diet in a 4×4 factorial arrangement.

The growth experiment was conducted in a recirculating water

system. Abalone were stocked at 30 animals for each rearing unit in a plastic basket ($20 \text{ cm} \times 20 \text{ cm} \times 10 \text{ cm}$) per glass aquarium ($45 \text{ cm} \times 25 \text{ cm} \times 35 \text{ cm}$). There were 16 treatments, and each treatment was conducted in three replicates. The feeding trial was run for 152 days.

Environmental Conditions of the Growth Experiment

During the 152-day growth experiment, water temperature was maintained at $18 \pm 1^\circ\text{C}$, salinity 31–35, pH 7.8–8.0. Dissolved oxygen was not less than 6 mg/L, and there were negligible levels of free ammonia and nitrite. Mineral concentrations in the seawater of the rearing system were determined by inductively coupled plasma-atomic emission spectrophotometer (ICP-OES: VISTA-MPX, VARIAN). Concentrations of Ca, P, Mg and Sr were 301.0, 0.5, 78.4 and 8.6 mg/L, and those of Cu, Zn, Fe, and Mn were 1.5, 8.2, 1.2 and 0.1 $\mu\text{g/L}$.

Preparation of Shell Samples

At the termination of the feeding trial, animals were not fed for 3 days. All the abalone were removed from the baskets, weighted, measured and counted. Then, abalone from each replicate were immediately frozen (-70°C). Shells were separated from the soft body, and then were cleaned under tap water to remove residual dirt. Subsequently, shells were kept in 5% NaOH for 7 h. After being washed completely with deionized water, the samples were air-dried for 24 h for the following analyses.

Scanning Electron Microscopy

To examine variation in crystalline ultrastructure, shells of abalone fed the diets with different levels of vitamin A and D were analyzed by SEM. Vertical sections of shells for SEM were prepared by fracturing. SEM was carried out on a JEOL JSM-840 instrument.

Mineralogy and Chemical Compositions

Mineralogical composition of the shells was investigated by x-ray diffractometry on powdered samples (Cohen & Branch 1992). The fraction of each type of crystal (aragonite, calcite, and dolomite) was expressed as a percentage.

Concentrations of Ca, P, Mg, Fe, Zn, Cu, and Sr in shells were determined by ICP-OES. Preparation of the shells was according to the method of Carriker et al. (1980).

Isolation and Purification of the Soluble Matrix Protein

The cleaned shells were crushed into a fine powder, then suspended in 3,500 molecular weight cutoff dialysis membranes (Cole-Parmer Instrument Co., USA) and dialyzed against 5% acetic acid containing 0.01% (w/v) sodium azide for 72 h at room temperature. This was followed by exhaustive dialysis against ultra-pure water for 48 h to remove the acetic acid. The resultant dialysate was centrifuged at $\times 10,000g$ for 30 min at 4°C . The supernatant containing protein SMP was collected and lyophilized for further analyses.

Vertical Polyacrylamide Gel Electrophoresis

Analysis of SMP was performed using the discontinuous buffer system. Electrophoresis of SDS-PAGE (18% separating gel and 4% stacking gel) was prepared with acrylamide:bisacrylamide and run in Mini-PROTEAN II equipment (Bio-Rad, Hercules, CA).

TABLE 1.

Composition of the basal diet.

Ingredient	g/100 g dry wt.
Casein (vitamin-free, Sigma Chemical, St. Louis, MO, USA)	25.0
Gelatin (Sigma Chemical, St. Louis, MO, USA)	6.0
Dextrin (Shanghai Chemical Co., Shanghai, China)	33.5
Sodium alginate (Shanghai Chemical Co., Shanghai, China)	20.0
SO/MFO (Food grade) ^a	3.5
Choline chloride (Shanghai Chemical Co., Shanghai, China)	0.5
Carboxymethylcellulose (Shanghai Chemical Co., Shanghai, China)	5.0
Mineral mix ^b	4.5
Cholecalciferol and retinol-free vitamin mix ^c	2.0
<i>Proximate analysis (means of triplicate)</i>	
Crude protein (%)	29.0
Crude lipid (%)	3.3
Ash (%)	9.6
Gross energy (kJ/g) ^d	18.8
Retinol (IU/kg)	32.8
Cholecalciferol (IU/kg)	25.6

^a Soybean oil and menhaden fish oil (1:1) with 0.001% ethoxyquin.

^b Mineral mix, each 1000 g of diet contained: NaCl, 0.4g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.0g; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 10.0g; KH_2PO_4 , 12.8g; $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$, 8.0g; Fe-citrate, 1.0g; Ca-lactate, 1.4g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 141.2 mg; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 64.8mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 12.4mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.4mg; KIO_3 , 1.2 mg; Na_2SeO_3 , 0.4 mg.

^c Cholecalciferol and retinol-free vitamin mix, each 1000 g of diet contained: thiamin HCl, 120 mg; riboflavin, 100 mg; folic acid, 30 mg; PABA, 400 mg; pyridoxine HCl, 40 mg; niacin, 800 mg; Ca pantothenate, 200 mg; inositol, 4000 mg; ascorbic acid, 4000 mg; biotin, 12 mg; B_{12} , 0.18 mg; menadione, 80 mg; vitamin E, 450 mg; ethoxyquin 400 mg.

^d Estimated with an XYR-1 bomb calorimeter.

USA). Samples (20 μ L) were added to wells (1-cm high). All the stock solutions were prepared by the methods described in the instruction manual (Bio-Rad, Hercules, CA, USA). Gel thickness was established with 1-mm spacers. Molecular weight (MW) marker was obtained from Bio-Rad (catalog number 161-0304). Other electrophoretic chemicals were from Amersham-Pharmacia (New Territories, Hong Kong, People's Republic of China). Procedures were carried out at room temperature. The power condition was 150 V, constant voltage setting. The usual run time was approximately 90 min. The method in combination with silver nitrate and stains-all (4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiocarbocyanine bromide), in the present study, was accepted to stain the gels (Goldberg & Warner 1997).

Amino Acids Analyses

To have enough samples for amino acid analyses, SMP from the 3 replicates in a treatment were pooled together. Sample (1 mg) of SMP was hydrolyzed *in vacuo* using 6N HCl for 24 h at 110°C and derivitized with dabsyl chloride (Kamp 1990). Analyses of derivitized amino acids were performed by reversed-phase HPLC (RP-HPLC) on an ODS (octadecylsilane) Hypersil column (HP; 250 \times 4 mm; 5 μ m). Eluant gradients with two solvent buffers were formed. The amount of acetonitrile (A) was gradually increased in 25 mM sodium acetate (pH 6.5) (B). Fifteen percent A was increased to 25% in 10 min, 40% in 20 min, 70% in 10 min, and held at 70% for 5 min before returning to 15% in 5 min. Tryptophan was lost during hydrolysis, and cystine coeluted with a reagent peak during amino acid separation. Therefore, tryptophan and cystine values are not reported. Values reported as Glx included both glutamate and glutamine, because glutamine was converted to glutamate during the analysis. Values for Asx included aspartate and asparagines, because asparagine was converted to aspartate.

Statistical Analysis

All percentage data were square-root arcsine transformed prior to analysis. Data were submitted to 2-way analysis of variance using the STATISTICA™ package. When significant differences ($P < 0.05$) were found, means were compared using the Tukey's test.

RESULTS

Scanning Electron Microscopy

There are no variations in shell ultrastructure of *H. discus hannai* fed the diets with different levels of vitamin A and D. Crystals in the shell could be divided into two layers from the outer to the inner shell surface; the prismatic layer and the nacreous layer. The former was composed of calcite. The latter was composed of tabular blocks of aragonite, and aragonitic tablets resemble a brick wall (Fig. 1). Growth line was found in the shells. It was continuous through the nacre (Fig. 2).

Mineralogy

Contents of aragonite, calcite, and dolomite in shell are given in Table 2. Aragonite was the dominant type of crystal. Although vitamin A significantly influenced contents of aragonite (91.90% to 96.39%) and calcite (1.72% to 5.25%), there was no obvious trend. Shell dolomite content (1.39% to 2.85%) was not significantly influenced by vitamin A.

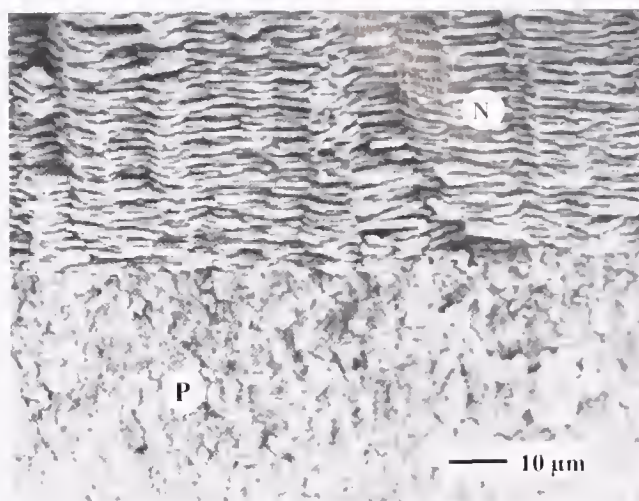


Figure 1. Vertical fracture-plane of the shell composed of calcitic prisms (P), and the succeeding nacreous layer (N). $\times 1,600$.

In the case of hypo- or hyper-vitamin A supplement (0 or 1×10^6 IU/kg), dietary vitamin D significantly increased shell calcite content. When dietary vitamin A supplements ranged from 1×10^3 to 1×10^5 IU/kg, vitamin D significantly decreased the content of calcite in shell. There were no significant effects of vitamin D on both aragonite and dolomite contents.

Significant interaction between vitamin A and D was found on contents of aragonite and calcite in the shell. When dietary vitamin A supplements ranged from 1×10^3 to 1×10^5 IU/kg, both vitamin A and D increased shell aragonite content, but decreased shell calcite content. Such significant interaction was not found with shell dolomite content.

Chemical Composition of Shells

Concentrations of Ca, P, Mg, Fe, Zn, Cu, and Sr in shell are given in Table 3. Vitamin A had significant effects on concentrations of Ca, P, Fe, and Cu. When dietary vitamin A levels ranged from 1×10^3 to 1×10^6 IU/kg it increased shell Ca concentration regardless of the dietary vitamin D supplements. Concentrations of

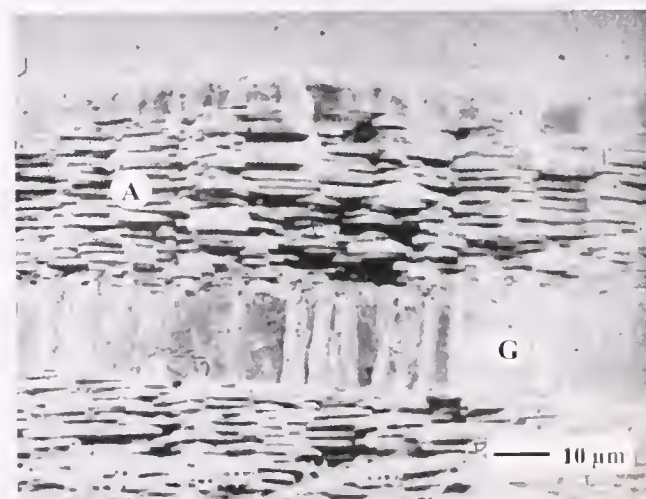


Figure 2. Nacre of shell and growth line. Nacre is composed of tabular blocks of aragonite (A), and growth line (G) is a deposit of calcium. $\times 1,600$.

TABLE 2.

Contents of aragonite, calcite and dolomite in the shell of *Haliotis discus hannai* fed the diets with different levels of vitamin A (A) and D (D) for 152 days.

Dietary Supplement		Aragonite	Calcite	Dolomite	C/A *
A (IU/kg)	D (IU/kg)	(%)	(%)	(%)	(%)
0	0	95.36 ^{ab}	2.90 ^{ef}	1.73	3.04 ^d
	500	94.39 ^{bcd}	3.05 ^{ef}	2.56	3.23 ^d
	1,000	93.66 ^{bcd}	3.76 ^{bcd}	2.58	4.01 ^{bcd}
	5,000	92.75 ^{de}	4.59 ^{abcd}	2.66	4.95 ^{abc}
1,000	0	91.90 ^e	5.25 ^a	2.85	5.71 ^a
	500	92.79 ^{de}	5.07 ^a	2.14	5.47 ^a
	1,000	94.12 ^{bcd}	3.38 ^e	2.51	3.59 ^d
	5,000	94.17 ^{bcd}	3.33 ^e	2.50	3.54 ^d
100,000	0	93.77 ^{bcd}	4.74 ^{ab}	1.49	5.05 ^{ab}
	500	94.13 ^{bcd}	3.87 ^{bcd}	2.00	4.11 ^{bcd}
	1,000	94.91 ^{abc}	3.70 ^{bcd}	1.39	3.89 ^c
	5,000	96.39 ^a	1.72 ^g	1.89	1.79 ^e
1,000,000	0	94.97 ^{abc}	3.52 ^{de}	1.51	3.71 ^d
	500	94.74 ^{abc}	3.68 ^{bcd}	1.57	3.89 ^c
	1,000	94.57 ^{abcd}	3.89 ^{bcd}	1.54	4.12 ^{bcd}
	5,000	93.39 ^{cde}	4.68 ^{abc}	1.94	5.01 ^{abc}
ANOVA					
A		0.0000	0.0001	0.1158	0.0000
D		0.2955	0.0008	0.7243	0.0040
A × D		0.0000	0.0000	0.8459	0.0000
Pooled s.e.		1.2222	0.9521	0.7818	1.0257

^{a-e} Means in the same column sharing a common superscript letter were not significantly different ($P > 0.05$) as determined by Tukey's test.

* C/A (%) = (calcite/aragonite) × 100.

the shell Fe in treatments with 1×10^5 and 1×10^6 IU/kg dietary vitamin A were significantly higher than those in treatments with 0 or 1×10^3 IU/kg dietary vitamin A.

Vitamin D had no significant effects on concentrations of P, Mg, Fe, Zn, and Sr in the shell. When dietary vitamin A ranged from 1×10^5 to 1×10^6 IU/kg, vitamin D significantly increased shell Ca concentration. Vitamin D significantly elevated the shell Cu concentrations at all dietary vitamin A levels.

Interaction between vitamin A and D were found significant on concentrations of Ca and Cu in shell, not on other analyzed minerals. This interaction significantly increased concentrations of Ca and Cu in shell.

SDS-PAGE

The SDS-electrophoretic separation of the SMP gave five bands (Fig. 3). The gel was somewhat overstained to help visualize minor components. Preparations of SMP from the different shells of abalone fed the diets with different levels of vitamin A and D showed a similar staining profile. Four distinct bands were found at 36.6, 30.9, 24.5, and 19.7 kDa respectively. A heavy stained region was labeled *a*.

Amino Acid Compositions

Apparent amino acid contents of the soluble matrix protein (SMP) from the shell are given in Table 4. The dominant amino acids in shells were Asx, Ser, Gly, and Phe. Meanwhile, Pro and Met were not detected in shells. Vitamin D increased the ratio of

acidic/basic amino acids [(Asx + Glx)/(Lys + Arg + His)], in the case of deficient (0 IU/kg) or excessive (1×10^6 IU/kg) dietary vitamin A supplement. When dietary vitamin A supplements ranged from 1×10^3 to 1×10^5 IU/kg, vitamin D decreased those ratios in shell.

DISCUSSION

To our best knowledge, the present study is the first report on the effects of vitamin A and D on shell biomineralization in molluscs. The results showed that various levels of the two vitamins in diets did not change the ultrastructure or the soluble matrix protein (SMP) components in the shell of *H. discus hannai*. However, mineralogical and chemical compositions in shells were significantly influenced by dietary vitamin A and D. In previous study, it was also demonstrated that mineralogical and chemical compositions were very important to investigate the effects of dietary guaiacol and Zn on shell biomineralization in *H. discus hannai* (Mai & He 2000, Mai et al. 2003). It is suggested that changes in mineralogical and chemical compositions in the shell are responsive indicators to study the relationship between nutrients and shell biomineralization in abalone.

The role of vitamin A in bone mineralization has been well established. Low doses of vitamin A induce expression of the alkaline phosphatase (AKP), osteonectin, and osteopontin genes. As a result, massive hydroxyapatite is deposited. The mechanisms by which vitamin A induces changes in mineralization-related gene expression in chondrocytes is possibly the temporal expression of nuclear retinoic acid receptor (RAR) isoforms (Iwamoto et al. 1993). Overdoses of vitamin A cause abnormalities in bone mineralization of larval flounder. These bone deformities were due to hypervitaminosis induced by excessive vitamin A that accelerated chondrocyte maturation (Dedi et al. 1995). In the present study, vitamin A significantly increased concentrations of Ca and P in the shell. Given that AKP plays an important role in metabolism of Ca and P (Norman et al. 1970, Birge & Avioli 1981), results mentioned earlier are suggested to relate to AKP activity in abalone. In our previous study, it was demonstrated that AKP activities in the viscera of abalone increased with dietary vitamin A levels (unpublished data). At present, it is difficult to identify whether vitamin A elevates expression of the AKP gene in abalone, because there are not enough direct data. Further study is needed to make the related mechanism clear.

Vitamin D can stimulate intestinal Ca and P intake and can lead to hypercalcemia and hyperphosphatemia in walking catfish *Clarias batrachus* and American eels *Anguilla rostrata* (Swarup & Srivastav 1982, Fenwick et al. 1984). Vitamin D did not significantly influence the concentrations of Ca and P in the bone of the blue tilapia *Oreochromis aureus* (O'Connell & Gatlin 1994). These findings indicate that the role of vitamin D in calcium metabolism of fish is not well established. In the present study, dietary vitamin D significantly increased concentrations of Ca and P in shell. These results are in agreement with those from a previous study (Zhou, pers. comm.). Given that AKP is involved in the metabolism of Ca and P (Norman et al. 1970, Birge & Avioli 1981), changes of AKP activity could be responsible for Ca and P concentrations in shell in the present study. It was demonstrated in our previous study that AKP activities were increased with dietary vitamin D (unpublished data). Vitamin D also elevated bone-specific AKP activity in vertebrates (Spiess et al. 1986). Combin-

TABLE 3.

Concentrations of Ca, P, Mg, Fe, Zn, Cu and Sr in the shell of *Haliotis discus hannai* fed the diets with different levels of vitamin A (A) and D (D) for 152 days

Dietary Supplement		Ca	P	Mg	Fe	Zn	Cu	Sr
A (IU/kg)	D (IU/kg)	(g/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(g/kg)
0	0	202.67 ^c	80.06 ^{cd}	296.79	405.67 ^b	53.37	0.49 ^c	1.81
	500	196.33 ^c	80.46 ^{cd}	298.79	405.71 ^b	54.44	0.50 ^c	1.84
	1,000	200.67 ^c	81.71 ^{bcd}	300.00	402.33 ^b	53.61	0.51 ^c	1.90
	5,000	200.51 ^c	82.30 ^{bcd}	295.88	410.28 ^b	58.35	0.52 ^c	1.84
1,000	0	201.33 ^c	80.12 ^{cd}	299.12	402.36 ^b	54.68	0.56 ^c	1.87
	500	201.67 ^c	80.52 ^{cd}	297.75	407.30 ^b	55.59	0.56 ^c	1.93
	1,000	199.67 ^c	82.40 ^{bcd}	308.65	414.00 ^b	54.07	0.55 ^c	1.86
	5,000	200.67 ^c	82.64 ^{bcd}	302.20	401.67 ^b	53.98	0.50 ^c	2.02
100,000	0	205.33 ^{bc}	81.20 ^{cd}	303.36	539.22 ^a	53.07	0.61 ^c	1.95
	500	208.21 ^{bc}	86.71 ^{abcd}	304.94	543.75 ^a	53.80	0.66 ^{bc}	1.87
	1,000	224.00 ^{abc}	89.43 ^{ab}	300.63	539.66 ^a	52.02	0.69 ^{bc}	2.03
	5,000	243.03 ^{ab}	91.87 ^a	307.07	561.20 ^a	57.23	1.04 ^a	1.94
1,000,000	0	205.70 ^{bc}	80.58 ^{cd}	304.27	562.35 ^a	57.59	0.57 ^c	1.87
	500	211.39 ^{bc}	86.77 ^{abcd}	299.26	561.58 ^a	58.29	0.62 ^{bc}	1.96
	1,000	229.60 ^{abc}	91.13 ^a	304.54	564.31 ^a	59.05	0.62 ^{bc}	1.97
	5,000	255.05 ^a	91.99 ^d	312.26	578.83 ^a	60.45	0.83 ^{ab}	1.93
ANOVA								
A		0.0000	0.0016	0.7926	0.0000	0.2287	0.0000	0.6437
D		0.0006	0.0597	0.4639	0.5182	0.2767	0.0000	0.8578
A × D		0.0206	0.5728	0.9544	0.9348	0.9534	0.0002	0.9888
Pooled s.e.		19.7782	5.8589	10.6968	78.0254	4.1823	0.1520	0.1846

^{a-d} Means in the same column sharing a common superscript letter were not significantly different ($P > 0.05$) as determined by Tukey's test.

ing these findings, it is suggested that vitamin D, as well as vitamin A, stimulate AKP activity, subsequently increasing the deposition of Ca and P in shell of *H. discus hannai*.

It has been demonstrated that the soluble matrix proteins (SMP) play important roles in the control of CaCO_3 crystal polymorphism phase switching and orientation in the mollusc shell (Belcher et al. 1996, Falini et al. 1996). Furthermore, aragonitic and calcitic SMP determine the nucleation and growth of aragonite and calcite, respectively (Belcher et al. 1996). Kawaguchi and Watabe (1993) reported high ratios of acidic/basic amino acids (50.1) in the calcitic prismatic layer soluble matrix (SM) of the American oyster *Crassostrea virginica*. Data calculated from some other studies shows that the ratio of acidic/basic amino acids (14.9) in calcitic shell of the Antarctic scallop *Adamussium colbecki* (Halloran & Donachy 1995) was greater than that (3.4) in aragonitic shell of the freshwater snail *Biomphalaria glabrata* (Marxen & Becker 1997). In the present study, trends of the ratio of calcite/aragonite in the shell, changing with different dietary vitamin A and D, were in agreement with those of the ratio of acidic/basic amino acids [(Asx + Glx)/(Lys + Arg + His)] in SMP. In our previous study, we found that ratios of acidic/basic amino acids, as well as ratios of calcite/aragonite in the shell of *H. discus hannai*, were significantly increased with dietary zinc levels (Mai et al. 2003). Results from the present study, and previous studies on other molluscs discussed earlier, may imply that SMP from calcitic shell was more acidic than that from aragonitic shell. It is suggested that the most likely way for vitamin A and D to influence the ratio of calcite/aragonite is to change the concentrations of calcitic and aragonitic SMP in shell. It has been well established in vertebrates that the most important way for vitamin A and D to influence the mineralization of bone and tooth is to regulate the gene expression of proteins

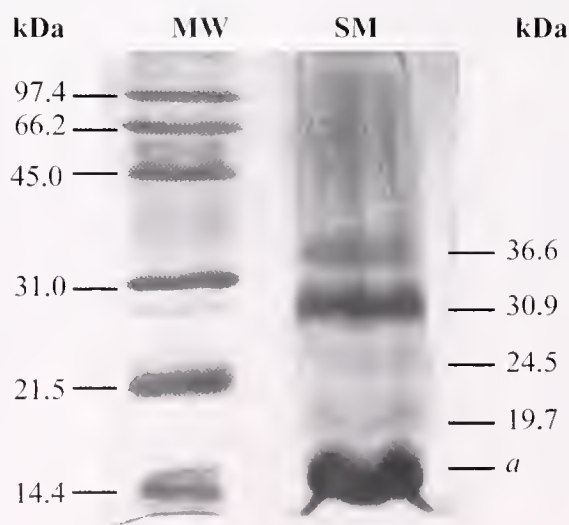


Figure 3. Stains-all and silver staining of the soluble matrix protein (SMP) from the shell of *H. discus hannai* fed the diets with different levels of vitamin A and D after electrophoresis. Because all the SMP preparations of shell show a similar pattern of bands, here only a representative is presented. Samples (20 μL) are loaded and electrophoresed on 18% acrylamide:bisacrylamide gels and stained with the Stains-all/silver protocol. Numbers on the left margin indicate the positions of molecular weight (MW) markers. Numbers and letter on the right margin are designations assigned to the different SMP.

TABLE 4.

Apparent amino acid contents of the soluble matrix protein (SMP) from the shell of *Haliotis discus hannai* fed diets with different levels of vitamin A (A) and D (D) for 152 days^a

Dietary Supplement		Amino Acid (Mole %)																
A (IU/kg)	D (IU/kg)	Asx	Glx	Ser	Thr	Gly	Ala	Pro	Val	Arg	Met	Ile	Leu	Phe	Lys	His	Tyr	A/B ^b
0	0	30.5	4.3	10.2	0.6	20.4	0.9	ND	3.8	3.5	ND	1.6	1.7	11.6	7.5	1.5	2.3	2.8
	500	30.8	5.0	9.5	0.6	20.6	0.8	ND	4.6	3.0	ND	1.5	1.3	11.3	7.3	1.5	2.4	3.0
	1,000	30.3	4.6	9.5	0.7	21.0	0.8	ND	4.2	3.2	ND	1.5	1.4	11.5	7.1	1.4	2.2	3.0
	5,000	31.0	4.7	9.2	0.4	20.7	0.6	ND	5.3	3.5	ND	1.2	1.6	11.7	7.0	1.2	2.1	3.1
1,000	0	30.6	4.0	9.7	0.6	19.6	0.5	ND	6.4	3.6	ND	1.5	1.5	11.2	7.0	0.8	2.3	3.0
	500	30.9	4.8	10.0	0.8	20.0	0.8	ND	3.2	3.8	ND	2.0	1.5	11.5	7.2	1.2	2.7	2.9
	1,000	31.0	4.3	9.2	0.6	20.8	1.0	ND	3.5	3.5	ND	1.6	1.7	11.4	7.1	1.5	2.5	2.9
	5,000	31.0	4.1	9.1	0.7	20.9	0.6	ND	4.7	3.5	ND	1.2	1.3	11.7	7.2	1.4	2.3	2.9
100,000	0	31.5	4.8	9.2	0.6	22.8	0.6	ND	2.9	3.7	ND	1.7	1.2	11.6	7.1	1.2	2.1	3.0
	500	30.2	5.0	9.2	0.6	20.0	0.7	ND	4.0	4.0	ND	1.2	1.7	11.5	7.5	1.5	2.6	2.7
	1,000	30.8	4.3	9.8	0.6	20.4	0.7	ND	3.9	3.8	ND	1.5	2.0	11.2	7.2	1.5	2.3	2.8
	5,000	30.7	4.0	9.0	0.6	19.6	0.9	ND	6.1	3.4	ND	1.3	1.2	11.4	7.1	1.6	2.2	2.9
1,000,000	0	30.2	5.1	9.6	0.7	20.7	0.8	ND	4.3	3.0	ND	1.8	1.3	11.6	7.3	1.6	2.0	3.0
	500	30.0	5.0	9.3	0.6	21.5	0.7	ND	5.2	3.0	ND	1.3	1.0	11.8	7.0	1.3	2.1	3.1
	1,000	30.1	4.8	9.4	0.6	21.0	0.7	ND	5.1	3.6	ND	1.7	1.4	11.2	7.2	1.2	2.0	2.9
	5,000	30.8	4.7	9.4	0.6	20.4	0.7	ND	5.1	3.6	ND	1.7	1.1	11.7	6.9	1.2	2.0	3.0

^a Because the amount of SMP from one replicate was not enough for analysis of amino acid composition, SMP from 3 replicates in a treatment were pooled as one sample for RP-HPLC.

^b A/B, acidic/basic amino acids [(Asx+Glx)/(Lys+Arg+His)]; ND, not detected.

involved in the formation of these mineralized tissues (Mahonen et al. 1990, Iwamoto et al. 1993, Luegmayr et al. 1998, Papagerakis et al. 1999). It is still unclear if vitamin A and D regulate the gene expression of SMP. Further work will be necessary to better understand the relationship between the two vitamins and SMP, and to investigate the molecular mechanisms of shell biomineralization in *H. discus hannai*.

ACKNOWLEDGMENTS

This study was financially supported by grant No. 30200215 from the National Natural Science Foundation of China (NNSFC) and grant No. 2001AA628080 and No.2004AA628100 from the National High Technology Research and Development Program of China (863 Program).

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SHELL MICROSTRUCTURE, MINERALOGY AND *IN VITRO* CRYSTALLIZATION STUDIES ON THE SHELL SOLUBLE MATRIX OF ABALONE, *HALIOTIS DISCUS HANNAI* INO

JIANMIN ZHAO, WENBING ZHANG, KANGSEN MAI,* WEI XU, ZHIGUO LIUFU, HONGMING MA, QINGHUI AI, AND BEIPING TAN

The Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, Qingdao 266003, People's Republic of China

ABSTRACT The shell microstructure of the abalone, *Haliotis discus hannai* Ino, was studied using scanning electron microscopy. The shell consists of the periostracum, prismatic, and nacreous layers with calcite in the outer prismatic layer and aragonite in the inner nacreous layer. FTIR analysis of the prismatic powder showed characteristic spectra of calcite: 876 cm^{-1} (ν_2) and 710 cm^{-1} (ν_4), whereas aragonitic nacreous powder had characteristic spectra of 858 cm^{-1} (ν_2) and $712\text{--}700\text{ cm}^{-1}$ (ν_4). Another absorption area in the range $2520\text{--}2650\text{ cm}^{-1}$ in both nacreous and prismatic layers was assigned to HCO_3^- groups, residing in the mineral or at the organic mineral interface, which suggests a potential participation of HCO_3^- groups as an intermediate in shell formation. In the *in vitro* crystallization experiment, it was found that the soluble matrix from different shell layers were able to induce different crystals with special morphologies. Soluble matrix, isolated from the prismatic layer, was shown to induce globular calcite formation *in vitro*, whereas soluble matrix from the nacreous layer induced the formation of almost symmetrical, hedgehog-like, aragonitic aggregates. The regulating mechanism of the formation, morphologic development, and crystallography of minerals in abalone shell is discussed.

KEY WORDS: *Haliotis discus hannai*, shell, microstructure, mineralogy, soluble matrix, *in vitro* crystallization

INTRODUCTION

Mollusk shells are polycrystalline composites of calcium carbonate and ~1% to 5%, by weight, of proteins and glycoproteins (Kaplan 1998). The shell has a fracture strength of about 3000 times higher than that of the inorganic CaCO_3 . These properties depend on microstructural characteristic, crystal phase, grain morphology and aggregation, and also on crystallite orientation (Chateigner et al. 2000).

The underlying secret of the above phenomena is the organic matrix existing in the mollusk shells. The matrix of shells can be separated into 2 broad classes: the soluble matrix (SM) and the insoluble matrix (IM). Soluble matrix is generally acidic due to high mole fractions of Asp and Glu, which are considered to play important roles in crystal nucleation, crystal growth and inhibition, crystal polymorphism, and atomic lattice orientation (Weiner & Addadi 1991, Wheeler 1992, Falini et al. 1996, Belcher et al. 1996). On the contrary, the insoluble matrix has a relatively high proportion of nonpolar amino acids, which are believed to provide the organic matrix framework for calcium carbonate to deposit (Weiner & Traub 1980, Weiner 1986).

CaCO_3 has 3 known polymorphs: calcite, aragonite, and vaterite phases. The calcite and aragonite are stable polymorphs and observed in nature, whereas the vaterite is a metastable polymorph and rarely seen in biologic systems (Weiner & Addadi 1997). Addadi & Weiner (1985) demonstrated that the biomacromolecules from shell could exert control over the polymorphism of a particular mineral phase. Previous studies on *in vitro* crystallization have shown that soluble proteins, associated with a variety of calcium carbonate biominerals, are able to interact with calcite growth and affect a variety of crystal properties (Berman et al. 1988, Berman et al. 1993, Didymus et al. 1993, Wierzbicki et al. 1994, Aizenberg et al. 1994, Sims et al. 1995). Falini et al. (1996) showed that macromolecules extracted from the aragonite layer of mollusk shells induced nucleation of aragonite crystals under an appropriate microenvironment. Moreover, Belcher et al. (1996)

demonstrated that a 16-kDa soluble polyanionic protein is sufficient to allow the transformation from the calcite to the aragonite phase. However, the exact mechanisms by which the macromolecules control the polymorphism, in both biotic and abiotic systems, are not yet well understood.

The shells of abalone are, in some cases, exclusively calcitic (e.g., *Haliotis kamtschatkana*, *Haliotis rufescens*) or aragonitic (e.g., *Haliotis asinina*, *Haliotis glabra*), but in many species the shell contains both calcite and aragonite (e.g., *Haliotis tuberculata*) (Dauphin et al. 1989). The diversity of crystal compositions in the shell of abalone presents a unique and interesting model for exploring shell biomineralization. As results on the biologic mechanisms of biomineralization have been obtained previously on the abalone *Haliotis discus hannai* Ino, it can be considered as a model to study the mineralization, using an interdisciplinary approach (Mai et al. 2003).

Until now there remains a large gap between the understanding of chemical model systems and biochemical/physiologic processes that control shell formation. The aim of this study is to investigate the inorganic part of the calcium carbonate shell of *H. discus hannai* Ino and to begin to elucidate the roles of the soluble matrix from abalone shell on the *in vitro* crystallization of calcium carbonate.

MATERIALS AND METHODS

Scanning Electron Microscopy Observation of Abalone Shell

Samples were fractured from the growing margin of abalone shell (shell length: 8.5 ± 0.3 cm, body weight: 75.3 ± 3.2 g) and mounted on a stub, vacuum-coated with gold-palladium for 5 min in a Technics gold-sputter, and scanned using a scanning electron microscopy (scanning electron microscopy, JEOL-840). Different layers of shell were subject to x-ray diffraction on a Rigaku diffractometer, which used a $\text{Cu K}\alpha$ radiation and operated at 40 KV and 100 mA, set to run from 25° to 60° at a step size of 0.01° 2θ and a counting time of 5 sec.

*Corresponding author. E-mail: kmai@ouc.edu.cn

IR Spectrometry Analysis

All spectra were recorded at 4 cm^{-1} resolution with 64 scans with a strong Norton–Beer apodization on a Perkin–Elmer model 1600 Fourier transform IR spectrometer (FTIR), in the wave-number range $4000 - 600\text{ cm}^{-1}$. The system was purged and permanently maintained under nitrogen to reduce atmospheric CO_2 and H_2O absorption. A background spectrum was measured for pure KBr. Sample spectra were automatically ratioed against background to minimize CO_2 and H_2O bands. All samples and KBr were dried in an oven at 38°C overnight and then ground into fine powder in an agate mortar for 10 min.

Isolation of the Soluble Matrix Proteins

The nacreous layer and prismatic shell were separated by mechanical scraping, then were crushed and pulverized into fine powder, respectively. Powders were suspended in 3,500 molecular weight cutoff dialysis membranes (Cole–Parmer Instrument, USA) and dialyzed against 5% acetic acid containing 0.01% (w/v) sodium azide for 72 h at room temperature. This was followed by exhaustive dialysis against ultrapure water for 48 h to remove the acetic acid. The resultant dialysate was centrifuged at $\times 20,000g$ for 30 min at 4°C , which separated the supernatant (SM) and precipitated (IM) fractions. The supernatant was collected and lyophilized for subsequent experiments.

Crystal Growth Experiments

CaCO_3 crystals were grown on glass coverslips in a CaCl_2 solution. Briefly, 3 mL of 12 mM CaCl_2 solution was introduced into the wells containing the coverslips. The wells were covered

with aluminum foil with punctures and placed inside a closed desiccator for 2 days for crystal growth by the slow diffusion of gases released by the decomposition of ammonium carbonate placed inside the desiccator (Rajamani et al. 2003). To study the role of soluble matrixes from nacreous and prismatic shells on CaCO_3 crystallization, aliquots of protein ($10\text{ }\mu\text{g/mL}$) were introduced into each well. After 2 days, the coverslips were carefully lifted from the crystallization vessels, rinsed gently with Millipore water, and air dried at room temperature for further analysis.

Characterization of Synthesized Crystals

The morphologies of the crystals were observed under an Olympus BH-2 phase contrast microscope (Olympus) with a digital camera (Pixera, Pro150ES). The crystals were then scraped and characterized by FTIR.

RESULTS

Shell Structure of Abalone Shell

There are 3 layers sequentially from the exterior to the interior of the abalone shell (Fig. 1). An uncalcified layer, called the periostracum, covers the shell externally. The calcified layer is composed of 2 different polymorphs of calcium carbonate: calcite and aragonite. The majority of the calcite forms the prismatic layer of the shell. The aragonite, formed between the mantle and the prismatic layer, is deposited as rounded or polygonal oriented (001) tablets. These tablets are $\sim 0.2\text{--}0.3\text{ }\mu\text{m}$ thick and $\sim 2\text{--}10\text{ }\mu\text{m}$ wide. The tablets are arranged in columns to form a stack with their c-axis aligned in the growing margin.

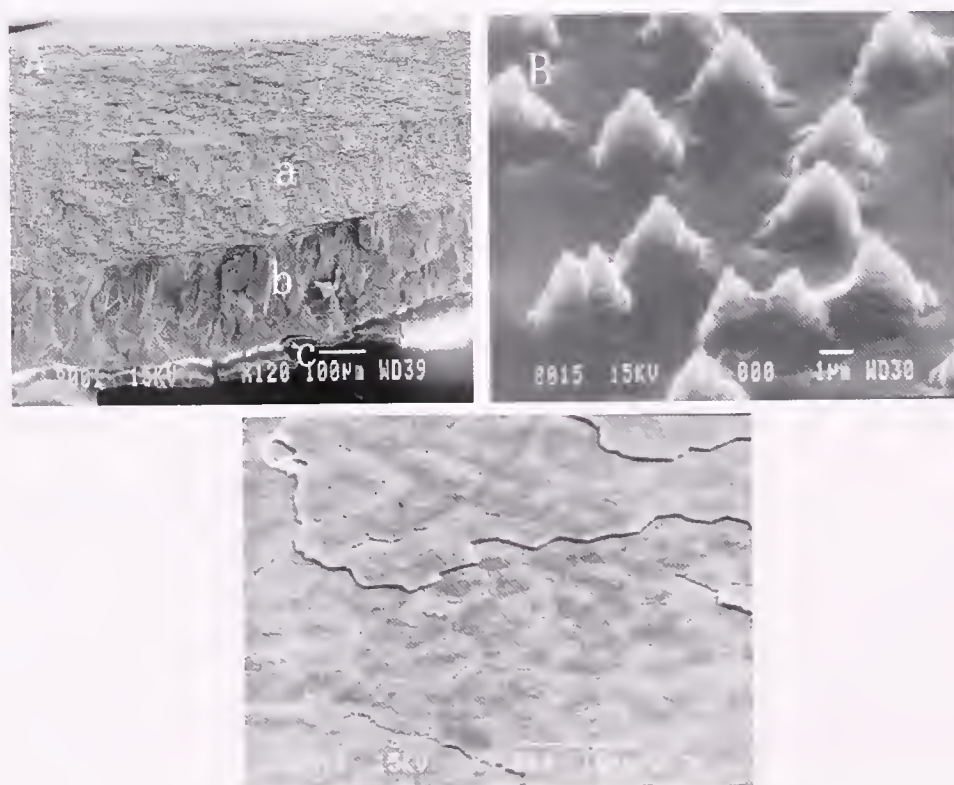


Figure 1. Scanning electron microscopy image of a vertical section of a fractured shell showing the shell structure of *Haliotis discus hannai* Ito. A. Three layers of the shell: nacreous layer (a), prismatic layer (b) and periostracum (c); B. the columnar structure resided in the growth margin of the shell; C. the polymorph of aragonitic tablets.

FTIR Analysis of Prismatic Powder and Nacre Powder

Figure 2 shows that both prismatic and nacreous powders were composed of organic matrix and mineral phase with numerous bands from 4000 cm^{-1} to 600 cm^{-1} . The spectra of calcite (prismatic powder) are characterized by 3 prominent absorption maxima at $1430\text{--}1450\text{ cm}^{-1}$ (ν_3), 876 cm^{-1} (ν_2) and 710 cm^{-1} (ν_4), and one minor band at $1012\text{--}1087\text{ cm}^{-1}$ (ν_1). The splitting of ν_4 , which is prevalent in the spectra of aragonite, is absent from calcite. The IR spectra of nacreous powder are characterized by 3 prominent absorption maxima at $1470\text{--}1490\text{ cm}^{-1}$ (ν_3), 858 cm^{-1} (ν_2), and $712\text{--}700\text{ cm}^{-1}$ (ν_4). Bands of lesser intensity appear at 1785 cm^{-1} and $1060\text{--}1080\text{ cm}^{-1}$ (ν_1). Another absorption area in the range of $2520\text{--}2650\text{ cm}^{-1}$ exists in both the nacreous and prismatic powders.

In vitro Crystallization of Calcium Carbonate

Figure 3 shows the morphologies and FTIR spectra of the crystals grown on the coverslips with or without soluble matrix from different origins. Figure 3a shows the morphology of crystals with-

out matrix protein supplementation. Rhombohedral calcite crystals were observed in the absence of matrix protein.

Figure 3b shows the morphology of crystals grown in the solution with prismatic soluble proteins and its FTIR spectra. The family of proteins from the prismatic layer induced a habit modification in calcite growth, yielding globular growth instead of the rhombohedra obtained in the absence of proteins. This may be the result of their relatively different rates of growth in the various directions. In the control treatment (Fig. 3a), calcite crystals show fuzzy step edges and the corners between step edges are sharp, whereas in the calcite grown in the presence of prismatic soluble matrix, step edges appear highlighted and corners between edges have become round.

In the nacreous soluble matrix supplemented solution, only aragonites are deposited, identified by the splitting of ν_4 in the FTIR spectra of deposited crystals as shown in Figure 3c. Hedgehog-like aggregates, with outward-oriented needles and their agglomerates, are formed.

DISCUSSION

In the present study, FTIR spectra showed an absorption area in the range of $2520\text{--}2650\text{ cm}^{-1}$ in both the nacreous and prismatic layer powder of *Halotis discus hannai*, indicating the presence of HCO_3^- . FTIR analysis of the nacre from *Pinctada maxima* also found HCO_3^- group and demonstrated that HCO_3^- group participated in the formation of calcium carbonate (Balmain et al. 1999). Studies have shown that nacrein, a 60 kDa protein isolated from the EDTA-soluble organic matrix of both nacreous and prismatic layers of *Pinctada maxima*, exhibits carbonic anhydrase activity (CA), suggesting that carbonic anhydrase acts to catalyze HCO_3^- to carbonate ions at the site of crystal growth, and that this is important for the formation of both aragonite and calcite (Miyamoto et al. 1996, Miyashita et al. 2002).

In the in vitro crystallization experiment, the soluble matrix from the nacreous layer induced the formation of aragonite, whereas the soluble matrix from the prismatic layer induced the deposition of calcite. It can be presumed that the selection of crystal polymorph, by cooperative interaction with the soluble matrix, showed perfect arrangement with the polymorph of calcium carbonate from which the soluble matrix had been isolated, suggesting that the polymorph selection of crystal is mediated by the differential production of these two different families of soluble matrix. The present results, and previous findings in other mollusk species (Belcher et al. 1996, Falini et al. 1996), have demonstrated that soluble matrix proteins, rather than insoluble ones, can control crystal polymorphism in vitro.

The deposition of less stable aragonite crystals was induced by the soluble matrix from the nacreous layer. Meanwhile, the screw character of calcite was deposited in the prismatic soluble matrix supplemented solution. These results indicate a strong interaction between the acidic groups present in the proteins and the growing crystals. It has been proposed that the interaction of proteins with specific crystal planes is governed by intrinsic stereochemical recognition of the macromolecules for specific molecular motifs exposed on certain crystal planes, which implies the stimulation of crystal formation at certain sites and relatively inhibition of the process at all other sites (Weiner & Addadi 1991, Mann et al. 1993). It is suggested that the nature and organization of functional groups at the surface of the proteins are crucial to achieve the desired selectivity in polymorph nucleation, as well as controlling the crystalline nature and morphology of the inorganic phase.

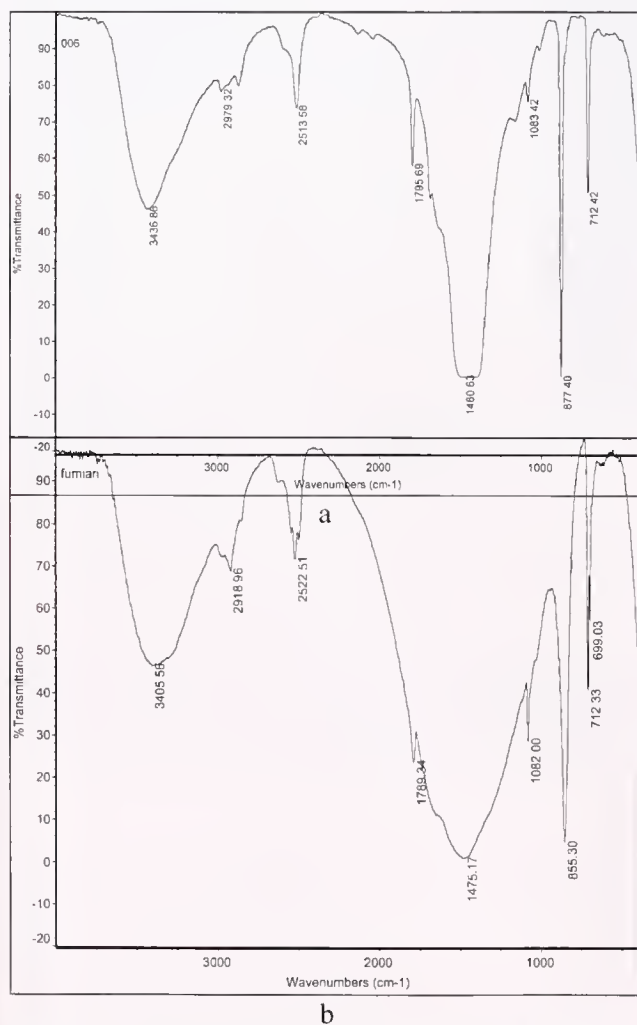


Figure 2. Fourier transform IR spectrometer (FTIR) spectrum (reflectance) of powdered prismatic (a) and nacre (b) (mineral and organic matrix) from *Halotis discus hannai* Ito.

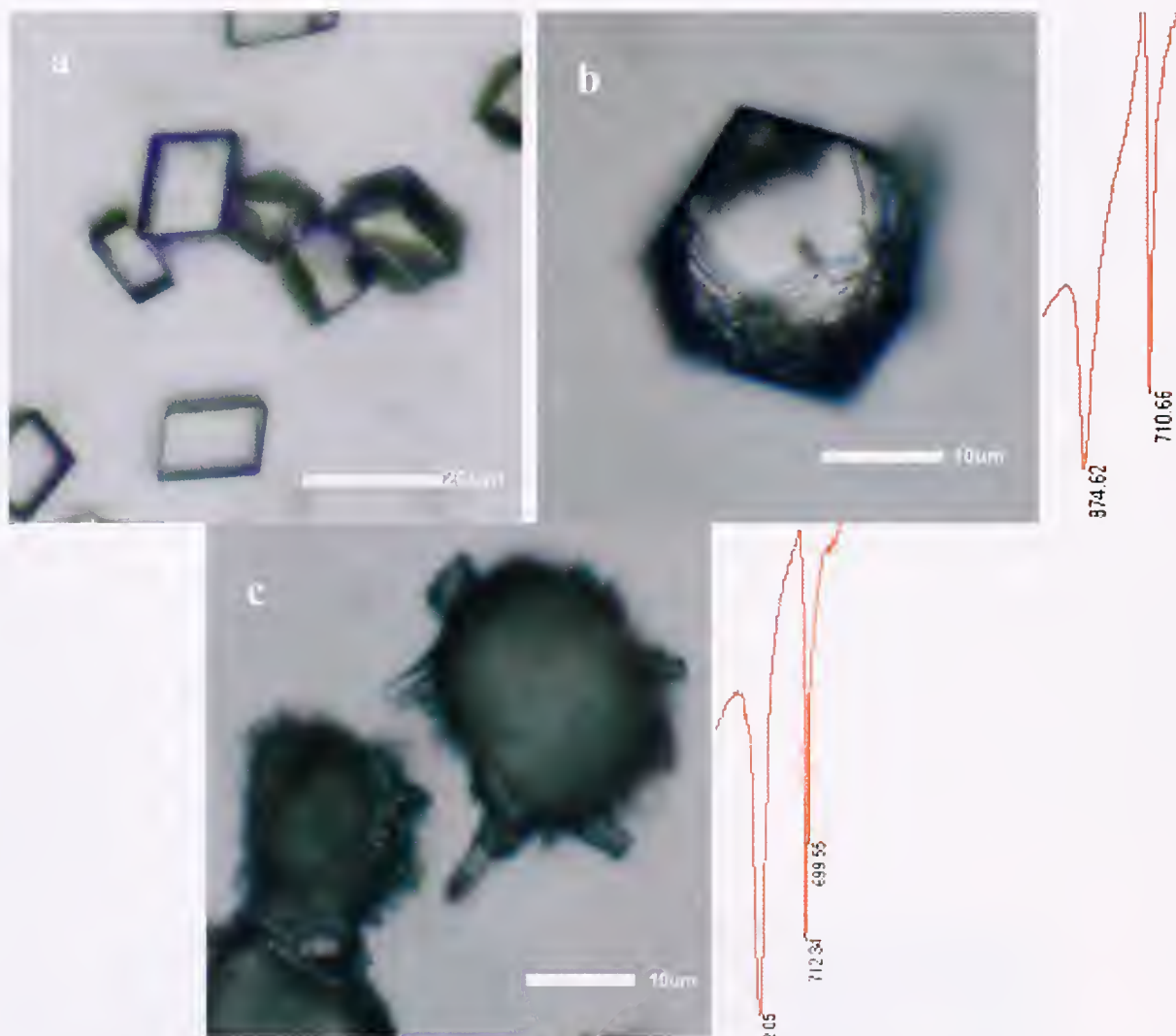


Figure 3. Optical micrographs show some of the salient features of crystals in control (a) and formed in the presence of 10 $\mu\text{g/mL}$ soluble matrix from prismatic layer (b) and nacreous layer (c) in the shell of *Haliotis discus hannai* Ito, respectively.

The weakness of the present study is that unpurified proteins have been applied in the in vitro mineralization experiments. It remains to determine which, if any, of the polyanionic proteins become occluded within the growing crystals, and with which crystallographic faces the individual proteins interact. To understand the underlying mechanisms of the interactions between proteins and mineral, it is essential to know the primary structure of the proteins involved. As a systematic work, we have purified the most dominate soluble protein in the nacre layer to

homogeneity and a molecular approach is underway (unpublished data).

ACKNOWLEDGMENTS

This study was financially supported by grant No. 2001AA628080 and No.2004AA628100 from the National High Technology Research and Development Program of China (863 Program) and grant No. 30200215 from the National Natural Science Foundation of China (NNSFC).

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MORPHOLOGICAL CHANGES IN THE RADULA OF ABALONE *HALIOTIS DIVERSICOLOR AQUATILIS* FROM POST-LARVA TO ADULT

TOSHIHIRO ONITSUKA,^{1*} TOMOHIKO KAWAMURA,¹ SATOSHI OHASHI,²
TOYOMITSU HORII,³ AND YOSHIRO WATANABE¹

¹Ocean Research Institute, The University of Tokyo, Nakano, Tokyo 164-8639, Japan; ²Nagasaki Prefectural Institute of Fisheries, Nagasaki, Nagasaki 851-2213, Japan; ³National Research Institute of Fisheries Science, Yokosuka, Kanagawa 238-0316, Japan

ABSTRACT The radula morphology of *Haliotis diversicolor aquatilis* was examined by SEM from postlarval to adult stages. In postlarvae of ~0.5 mm SL, a rachidian tooth (R), 2 pairs of lateral teeth (L1, L2), and 2–3 pairs of marginal teeth (M) per row were present. Only the number of marginal teeth increased to 12–13 pairs per row by 1.5 mm SL. An additional 2 pairs of lateral teeth (L3, L4) were added progressively from 1.5–2 mm SL. The L5 teeth were added at about 6 mm SL. Postlarvae with <2 mm SL had strongly curved rachidian and lateral teeth with clearance angles of ~15° to 20°. Each tooth had many serrations on the working edges. These structures of the teeth are probably suitable for collecting biofilm components such as extracellular mucus of diatoms. Postlarvae with >2.5 mm SL had higher clearance angles of 20° to 35°. Serrations on the working edges of rachidian and lateral teeth became progressively shallower. These changes suggest that the teeth are more suitable for grazing substrata to efficiently remove strongly attached diatoms. As abalone grew larger than 2 mm SL, the L3 and L4 teeth became longer than R, L1, and L2. The radula development of *H. diversicolor aquatilis* shown in this study is similar to that of *H. discus hannai*. However, it is suggested that the feeding transitions in the postlarval stage occur later in *H. diversicolor aquatilis* than in *H. discus hannai*. The morphology of juvenile and adult radula teeth of *H. diversicolor aquatilis* is clearly distinguishable from that of *H. discus hannai*. The sizes of L4 and L5 teeth of juvenile and adult *H. diversicolor aquatilis* were much smaller than L3 teeth, whereas sizes of L3–L5 teeth were not clearly different in *H. discus hannai*. This difference in radula morphology in juvenile and adult stages suggests that the main food items for juvenile and adult *H. diversicolor aquatilis* may be different from those of *H. discus hannai*.

KEY WORDS: abalone, radula, development, morphological changes, feeding, *Haliotis diversicolor*

INTRODUCTION

Haliotis diversicolor aquatilis Reeve 1846 is a commercially important abalone in Japan. However, the catch of this species has been declining probably because of over-fishing. Because reseed-ing of hatchery-reared juveniles into wild populations has been conducted in many places to enhance the stocks, ecologic studies of this species have been very limited, especially in the early life stages. Since 2001, we have started studying the reproduction and early life ecology of *H. diversicolor aquatilis* at Nagai in Sagami Bay, Japan. Natural recruitment of this species at Nagai seemed relatively high in comparison with those of other abalone species (*H. discus discus* Reeve 1846, *H. madaka* Habe 1979 and *H. gigantea* Gmelin 1791) in the same area, but growth and survival rates of postlarval *H. diversicolor aquatilis* was variable among sampling areas (Onitsuka et al. unpublished).

Quantity and quality of food are important factors affecting the growth and survival of postlarval abalone (Kawamura et al. 1998), and these factors have been suggested to be critical in controlling recruitment of *H. discus hannai* Ito 1953 (Kawamura et al. 2002). Feeding habits in the early life stages of abalone have been studied in detail for *H. discus hannai* in recent years (reviewed by Kawamura et al. 2002, Takami & Kawamura 2003), and three major transitions in feeding have been identified (Kawamura et al. 1998, Takami & Kawamura 2003). The transitions in feeding are suggested to be closely related to structural and functional changes in the radula during postlarval and juvenile stages (Kawamura et al. 2001). However, there is little information concerning the feeding habits of *H. diversicolor aquatilis*, and the diets in the early life stages are generally unknown, as well as for other many abalone

species. In addition, the radula morphology of *H. diversicolor aquatilis* has only been examined for adults (Wu 1991). In the present study, changes in radula morphology of *H. diversicolor aquatilis* are described from postlarva to adult. By comparing the radula development with that of *H. discus hannai* (Kawamura et al. 2001) we try to estimate the feeding habits of *H. diversicolor aquatilis* in postlarval and juvenile stages.

MATERIALS AND METHODS

Abalone Rearing

Larval *H. diversicolor aquatilis* were hatched in October 2000, September 2001, and August 2002 at the Nagasaki Prefectural Institute of Fisheries (Nagasaki, Japan). One-day-old larvae were transferred to culture tanks with plastic plates covered with naturally appeared benthic diatoms as settlement plates. Postlarvae that settled on the plates were reared continuously in sand-filtered running seawater at 20°C.

In this study, we defined the boundary between postlarval and juvenile *H. diversicolor aquatilis* as the size at which the first respiratory pore is sealed (~2.5 mm SL, Onitsuka et al. unpublished), following the definition for *H. discus hannai* by Takami (2002). We call individuals larger than 35 mm SL adults according to Oba (1964).

Radula Observation

Samples of postlarvae and juveniles were preserved in 5% formalin in seawater at intervals of 3 days from 10 to 25 days post-fertilization, and at 55 days and 163 days postfertilization in 2002. One, 3, 4, and 16-mo-old juveniles in 2001, 28-mo-old juveniles in 2000 were also preserved in 5% formalin in seawater. Radulae of postlarvae and small juveniles were removed with a pipette under

*Corresponding author. E-mail: oni2z@ori.u-tokyo.ac.jp

an inverted microscope following dissolution of tissues by soaking in sodium hypochlorite (0.6% Cl concentration; Wako Pure Chemical Industries Ltd, Osaka, Japan) for several minutes. Radulae were then serially pipetted through several distilled water baths to remove residual sodium hypochlorite. This procedure has been shown to preserve the structure of radula teeth (Kawamura et al. 2001). Abalone size represented by the longest shell length (SL) was measured individually before dissolution. Radulae of large juveniles and adults were dissected from preserved animals under a dissecting microscope after measuring shell length. The radulae were soaked in sodium hypochlorite to dissolve surrounding tissues attached to the radulae and then washed with distilled water to remove residual sodium hypochlorite. Because we could not get adult abalone from the hatchery, wild adult *H. diversicolor aquatilis* of 52.5–76.9 mm SL were caught at Nagai in 2002 as adult samples.

Radula length, width, number of transverse rows of teeth (Fig. 1A), and number of lateral and marginal teeth per row (except for individuals smaller than 6.5 mm SL) were determined using a monitor and digital camera system with an image analyzer, connected to an inverted microscope and a dissecting microscope. Radulae were then transferred to scanning electron microscope (SEM) stubs, laid flat with the teeth upward, and allowed to air dry before sputter coating with platinum for SEM observations.

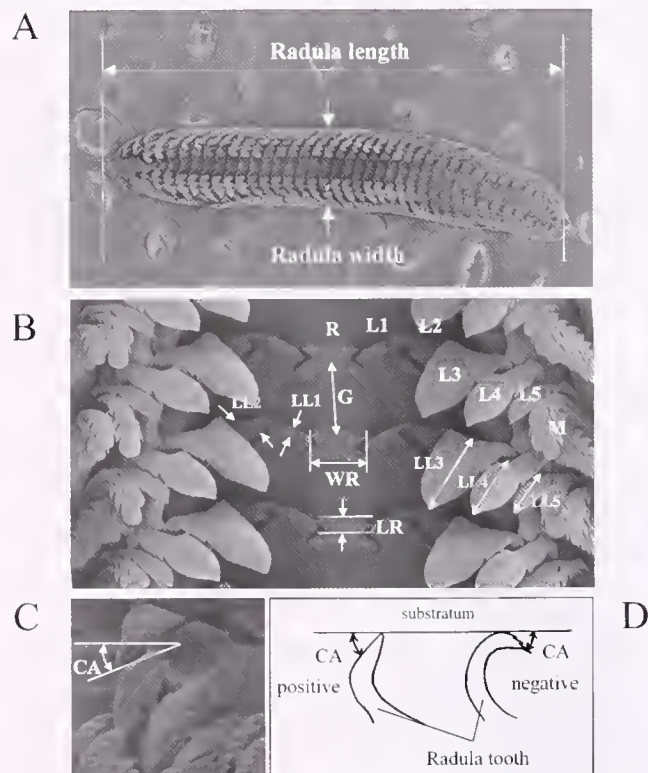


Figure 1. Scanning electron micrographs and an illustration describing radula morphology. (A) An entire radula showing length and width measurement. (B) Transverse rows of radula teeth. Tooth types: R = rachidian, L1–L5 = lateral teeth 1–5, M = marginal teeth. Measurements: WR = width of rachidian tooth, LR = length of rachidian tooth, LL1–LL5 = length of L1–L5 teeth, G = gap between rachidian teeth of adjacent transverse rows. (C) Side view of a row of radula teeth showing individual teeth. CA = clearance angle (positive). (D) A single tooth in profile illustrating positive (left) and negative clearance angle (right).

The length and width of rachidian teeth, lengths of the lateral teeth, gap between the rachidian teeth of adjacent transverse rows (Fig. 1B), and number of lateral and marginal teeth per row were measured using SEM photographs. Relative length of L4 and L5 teeth to L3 teeth was also calculated. The clearance angle of rachidian and lateral teeth (L1, L2; Fig. 1C, D) of postlarval and juvenile (<10 mm) radula, which was suggested to provide information on the function of radula teeth (Padilla 1985), was measured using the method by Roberts et al. (1999b). These measurements were done for five teeth (R, L1, L2) on a row located in the middle part of the whole radula. The clearance angles of older juvenile and adult radula were not measured because accurate measurements were obstructed by the overhanging marginal teeth. Measurements of the angles of rachidian teeth and inner lateral teeth of juveniles and adults could not be performed because they were located behind the lengthened outer laterals.

RESULTS

During the postlarval and juvenile period the radula underwent various morphologic developments before reaching the adult form. Most developments correlated more strongly with shell length than age (Table 1), so the following description relates primarily to shell length.

Overall radula length increased linearly with shell length (Fig. 2). However, the factors determining length changed as the abalone developed. During the early post larval period (<~0.8 mm SL), the number of transverse rows of teeth was the main factor for increasing radula length. The radula of postlarvae with ~0.5 mm SL contained 19 to 21 rows of teeth, and the number of rows increased to 22 to 26 at ~0.8 mm SL (Fig. 3B). Late postlarvae (0.8–2 mm) had 22 to 26 rows of teeth, therefore the increase in radula length reflected an increased gap between adjacent rows of teeth (Fig. 2) and an increased length of rachidian and lateral teeth (indicated by rachidian and L3 in Fig. 2). The number of rows of teeth started increasing again above a size of 2 mm (Figs. 3A, B). Above 1.5 mm SL the gap between rows of teeth increased rapidly (Fig. 2) as differentiation of the L3–L5 teeth developed. Therefore, the increase in radula length of abalone >2 mm SL was caused by increases in row number (Figs. 3A, B), the gap between rows of teeth and teeth length (Fig. 2).

The width of the radula also increased linearly as abalone grew (Fig. 4). The increase in radula width for postlarvae was mainly caused by the increase in the number of marginal (Fig. 5) and lateral teeth per row (Fig. 6). Postlarvae with ~0.5 mm SL contained the rachidian tooth (R), 2 pairs of lateral teeth (L1 and L2),

TABLE 1.

Correlation of radula variables with post-larval age and post-larval shell length. Post-larvae were chosen to include a range of sizes for each age. Data are Pearson's correlation.

Variable	Shell Length	Age	n
Overall length of radula	0.998	0.953	76
Overall width of radula	0.992	0.964	76
Number of rows of teeth on radula	0.979	0.943	76
Width of rachidian tooth	0.971	0.901	31
Length of rachidian tooth	0.937	0.899	31
Gap between adjacent rachidian teeth	0.977	0.944	31
Number of lateral teeth per row	0.934	0.886	31

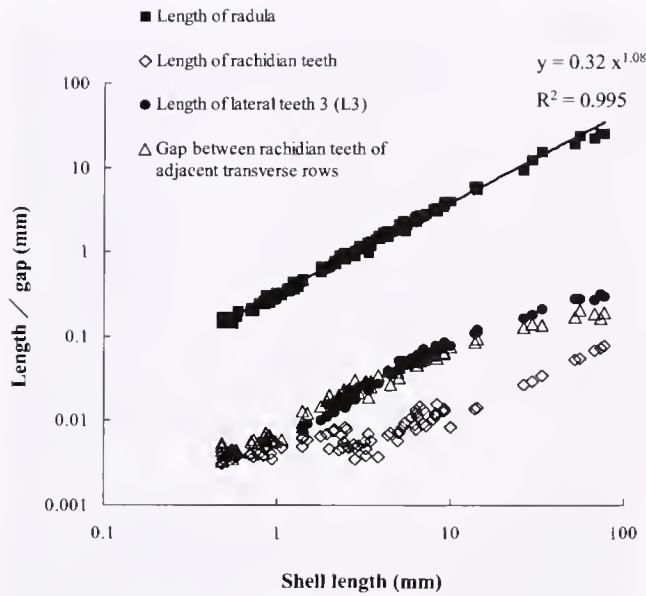
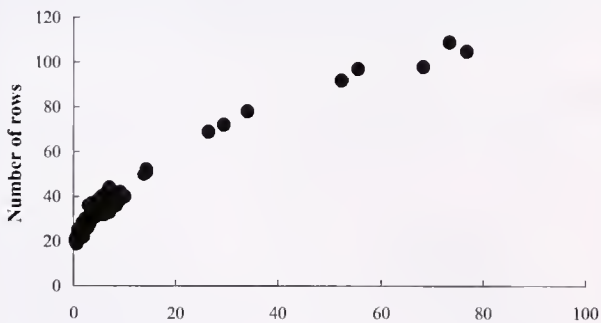


Figure 2. Increase in radula length with abalone shell length, from postlarva to adult. Graphs plot factors contributing to the change in radula length: the length of rachidian tooth, length of L3, and gap between rachidian teeth in adjacent transverse rows.

A



B

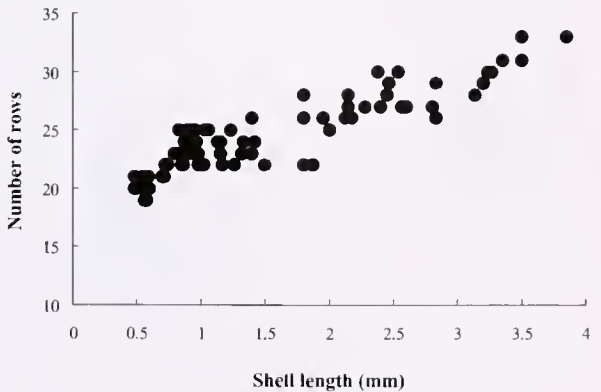


Figure 3. Relationship between abalone shell length (SL) and number of transverse rows. (A) Increase in number of transverse rows from postlarva to adult. (B) Number of transverse rows of postlarvae <4 mm SL.

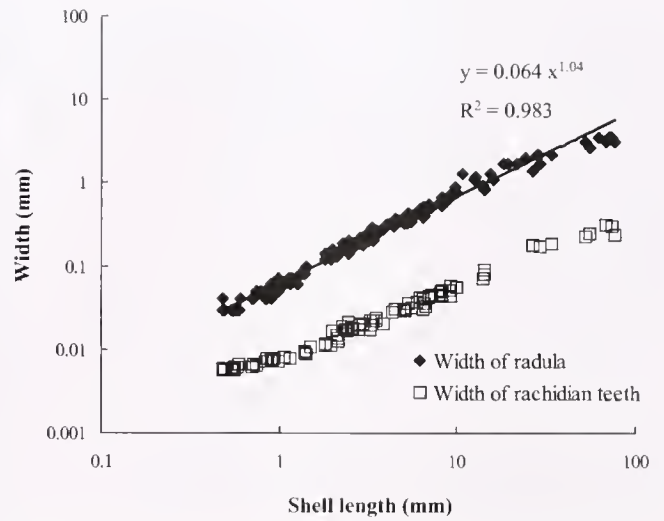


Figure 4. Increase in radula width with abalone shell length, from postlarva to adult. Graphs plot factor contributing to the change in radula width (i.e., the width of rachidian teeth [<77 mm SL]).

and 2 to 3 pairs of marginal teeth per row (Fig. 7A), although lateral and marginal teeth were not clearly differentiated. The number of marginal teeth per row increased progressively; postlarvae with shell lengths of 1.5, 6, and ≥ 30 mm had 12–13, 30–40, and 70–80 pairs of teeth respectively (Fig. 5). Postlarvae smaller than 1.5 mm SL contained only 2 pairs of lateral teeth (L1 and L2) (Fig. 7B). Two additional pairs of lateral teeth (L3 and L4) were added progressively in animals with ~ 1.5 mm to ~ 2 mm SL (Fig. 6). A pair of L5 was added in animals with about 6 mm SL, and at this size the adult complement of lateral teeth was completed (Fig. 6). The increase in width during juvenile stage was caused mainly by the increase in width of the individual teeth (illustrated by the width of the rachidian teeth in Fig. 4) and by the increase in the number of marginal teeth per row (Fig. 5). The increase in radula width of adults was caused by the increase in width of the individual teeth (Fig. 4).

Changes in the Morphology of Individual Teeth

The serrations on the working edges of the rachidian and lateral teeth became less pronounced as abalone grew. R, L1, and L2 teeth

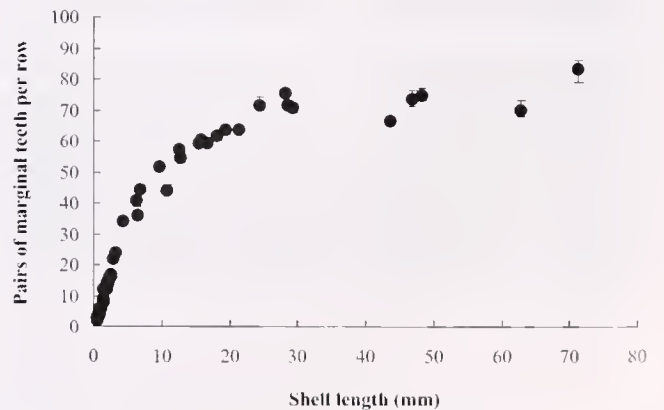


Figure 5. Relationship between abalone shell length (<70 mm SL) and number of pairs of marginal teeth in each transverse row. Each data point shows the mean \pm SE of 5 rows from 1 radula.

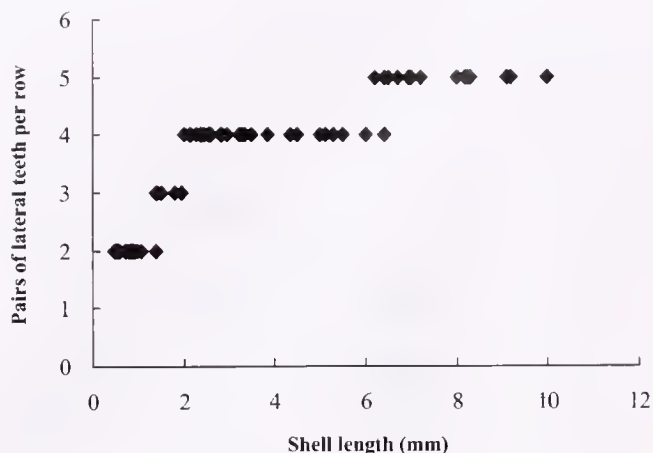


Figure 6. Relationship between abalone shell length (<10 mm SL) and number of pairs of lateral teeth in each transverse row.

initially had long, pointed serrations (Figs. 7A, B). In animals with more than 1 mm SL, these serrations became progressively shallower, first on R, and later on L1 and L2 as L3–L5 developed. By approximately 2 mm SL, nearly all serrations had disappeared from R, L1, and L2 teeth, but L3 and L4 retained serrations on their edges (Figs. 7D, E). All serrations disappeared from L3–L5 teeth in adult radula (Fig. 7F).

The L3–L5 teeth were already slightly longer than R, L1, and

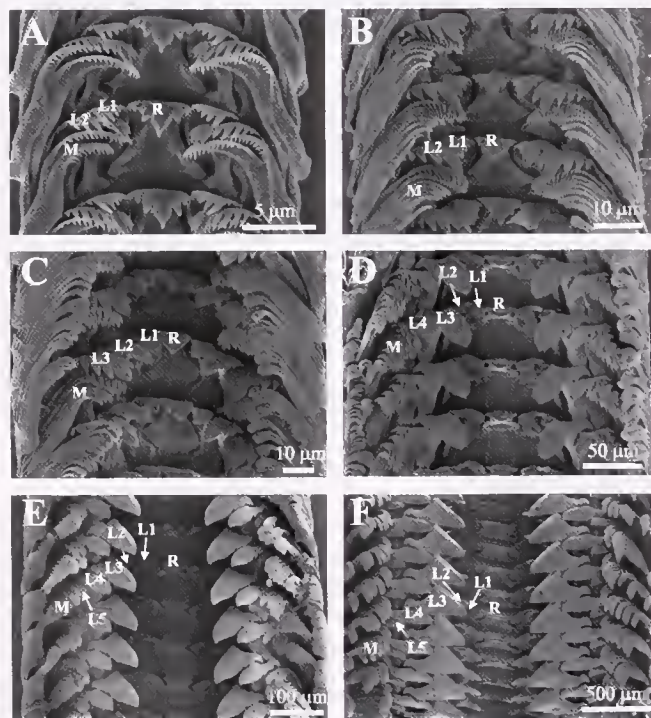


Figure 7. Scanning electron micrographs of the radula showing developmental stages. (A) Postlarva with 0.48 mm SL, 2 + 2 + R + 2 + 2. (B) Postlarva with 1.4 mm SL, 8 + 2 + R + 2 + 8. (C) Postlarva with 1.8 mm SL, 13 + 3 + R + 3 + 13. (D) Juvenile with 4.4 mm SL, 34 + 4 + R + 4 + 34. (E) Juvenile with 9.2 mm SL, ~50 + 5 + R + 5 + ~50. (F) Adult with 76.9 mm SL, ~90 + 5 + R + 5 + ~90. R = rachidian, L1–L5 = lateral teeth 1–5, M = marginal teeth. Radula formulae represent numbers of M + L + R + L + M (Voltzow 1994).

L2 when they were added, but difference became greater as abalone grew (Figs. 7D–F, Fig. 8A). This differentiation of the lateral teeth accompanied an increase in the gap between adjacent rows of teeth (Fig. 2). The L3–L5 teeth became pointed, similar to canine teeth, whereas the tip of R and L1 teeth became flat, similar to spades (Fig. 7F), during the juvenile and adult stages. The L2 teeth of adults were longer and much more pointed at the tip than R and L1, but much shorter than L3. The sizes of outer lateral teeth (L3–L5) were different in juvenile and adult radula. The relative lengths of L4 and L5 teeth to L3 teeth were 0.7 and 0.5 (Fig. 8B), although their shapes were similar (Figs. 7E, F). The size of L5 teeth was almost the same as marginal teeth, but much more pointed at the tip than marginals (Fig. 7F).

Marginal teeth were narrow and comb-like, with many fine serrations present from postlarva to adult (Fig. 7). The size of individual marginal teeth relative to the rachidian and lateral teeth became smaller, and the tip of marginal teeth became more round as abalone grew. The marginal teeth of adult abalone retained serrations on their edges, but the serrations became less pronounced near the tip (Fig. 7F).

Post-larval abalone smaller than about 2 mm SL had strongly curved rachidian and lateral teeth (Fig. 9A) with mean clearance angles of about -15° to 20° (Fig. 10), although the angle was variable within and between radulae. The clearance angle increased in the late postlarval stage between 2–2.5 mm SL, and the rachidian and lateral teeth of juveniles (>2.5 mm SL) had higher clearance angles of 20° to 35° (Figs. 9B, 10).

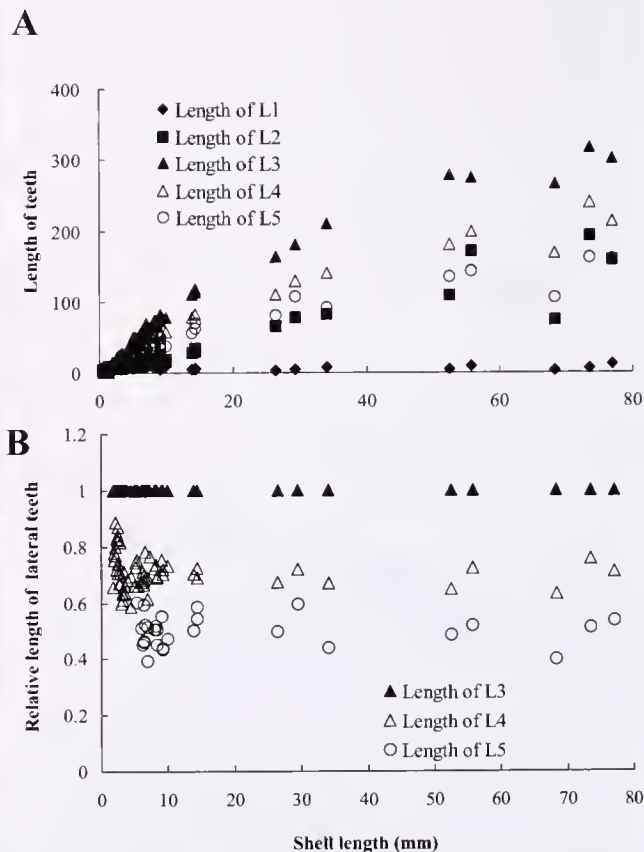


Figure 8. (A) Relationship between abalone shell length (<80 mm SL) and length of lateral teeth (L1–L5). (B) The relative length of L4 and L5 teeth to L3 teeth.

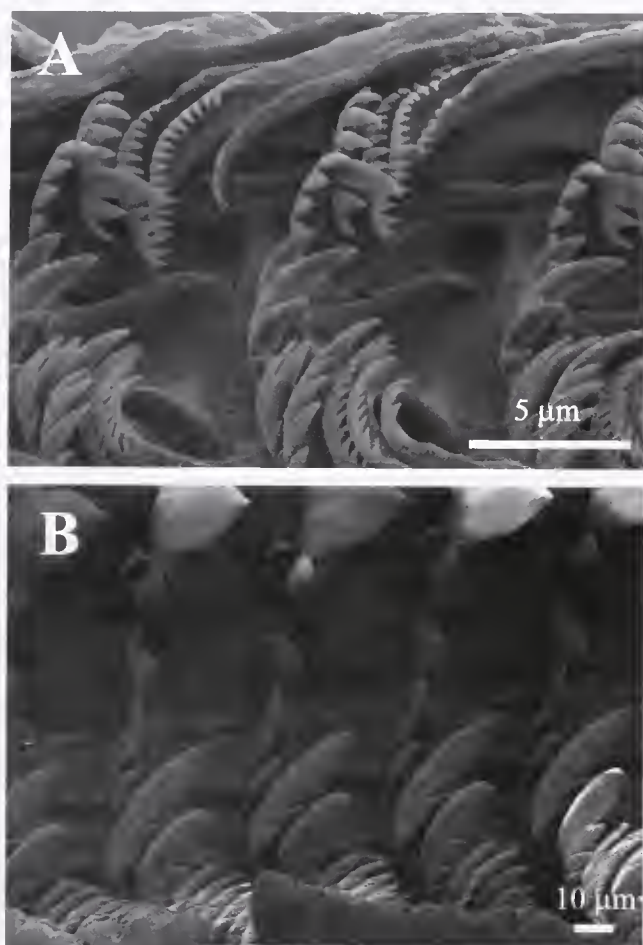


Figure 9. Scanning electron micrographs showing side views of abalone radula. (A) Strongly curved rachidian and lateral teeth of postlarvae (0.55 mm SL) with low clearance angles. (B) Straight-cusped radula teeth with higher clearance angles from a 4.5 mm SL juvenile.

DISCUSSION

Morphology of Adult Radula

Kawamura et al. (2001) showed that the development of the radula of *H. discus hannai* was remarkably similar to that of *H. iris*

Gmelin 1791 (Roberts et al. 1999b). The morphology of adult teeth was not clearly distinguishable between the two species. These two species are not genetically close (Lee & Vacquier 1995), but their feeding habits and growth patterns are similar (Kawamura et al. 1998). The radula teeth of adult *H. diversicolor aquatilis*, shown in this study, were easily distinguished from the radula of *H. iris* (Roberts et al. 1999b) and *H. discus hannai* (Kawamura et al. 2001). The size of outer lateral teeth (L3–L5) was apparently different in *H. diversicolor aquatilis*, L3 was the largest and L5 was the smallest (Figs. 7D–F, 8), while sizes of L3–L5 teeth were not clearly different in *H. iris* and *H. discus hannai*. Similar differences in the size of L3–L5 teeth were seen in tropical small abalone species *H. asinina* Linnaeus 1758, *H. ovina* Gmelin 1791 (Chitramvong et al. 1998), and *H. varia* Linnaeus 1758 (Wu 1991); but greater differences were observed in *H. diversicolor aquatilis*. The different morphologies of adult radula teeth may indicate different feeding habits among abalone species.

Development Process of Radula

The development process of the radula in *H. diversicolor aquatilis* was similar, but generally slower than in *H. iris* (Roberts et al. 1999b) and *H. discus hannai* (Kawamura et al. 2001). The initial increase in the number of teeth rows after metamorphosis was observed until the animal reached ~0.8 mm SL in *H. diversicolor aquatilis* (Fig. 3B), whereas in *H. iris* (Roberts et al. 1999b) and *H. discus hannai* (Kawamura et al. 2001) it was until ~0.5 mm SL. However, the size at which the row number started increasing again was ~2 mm SL in *H. diversicolor aquatilis*, which is smaller than the size of the animal with 4–5 mm SL reported for *H. discus hannai* (Kawamura et al. 2001). The mean postlarval size (the longest dimension of the larval shell) at metamorphosis of *H. diversicolor aquatilis* (257 μ m) is smaller than that of *H. discus hannai* (277 μ m) (Hayashi 1983). The remarkable increase in clearance angle of rachidian and lateral teeth was observed at 2–2.5 mm SL in *H. diversicolor aquatilis* (Fig. 10), whereas it is noticed at ~1 mm SL in *H. iris* (Roberts et al. 1999b) and *H. discus hannai* (Kawamura et al. 2001). The L5 teeth were added completing the adult complement of five pairs of laterals at ~6 mm SL in *H. diversicolor aquatilis* (Fig. 6), whereas at ~1.7 mm SL in *H. iris* (Roberts et al. 1999b) and at ~2 mm SL in *H. discus hannai* (Kawamura et al. 2001).

Estimated Transitions in Feeding

Three major transitions in feeding have been identified in *H. discus hannai* (Kawamura et al. 1998, Takami & Kawamura 2003), which appears to be closely related to radula development (Kawamura et al. 2001). The first transition is from lecithotrophy to particle feeding, around the time of metamorphosis. Postlarval *H. iris* (Roberts et al. 1999a) and *H. discus hannai* (Seki & Kan-no 1981, Kawamura & Takami 1995) begin feeding, within a day of the velum being shed, on the surface of crustose coralline algae (CCA) where larval abalone preferentially settle in the natural environment (e.g., Saito 1981, Morse & Morse 1984). However, postlarvae can grow up to ~0.4 mm SL without food during several days following metamorphosis (Roberts et al. 2001, Takami et al. 2000) by using their residual yolk supply as an energy source (Roberts et al. 2001, Takami et al. 2002). The early increase in the number of teeth rows was suggested to be related to the first transition in feeding, indicating active formation of new rows of

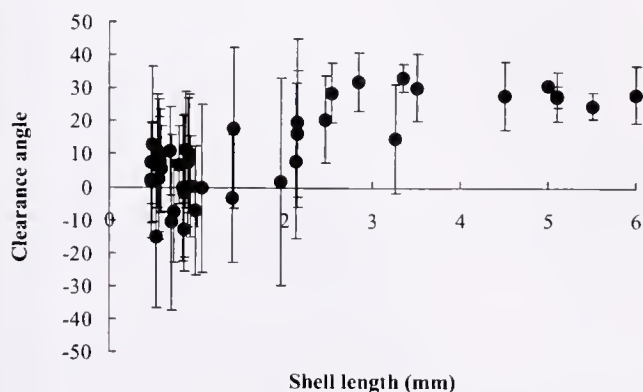


Figure 10. Relationship between teeth clearance angle and shell length of postlarva and juvenile *Haliotis diversicolor aquatilis*. Each data point shows the mean \pm SE of 5 teeth from 1 radula.

teeth prior to shedding anterior worn teeth during feeding (Kawamura et al. 2001). The greater size at which *H. diversicolor aquatilis* concluded the initial addition of teeth rows (0.8 mm SL) may indicate longer contribution of residual yolk reserves as an energy source after metamorphosis for this species. After the initial increase, the number of rows remained relatively constant until ~2 mm SL and then started increasing again (Fig. 3B). In *H. discus hannai* the number of rows was relatively constant between ~0.5 mm and 5 mm SL (Kawamura et al. 2001), which is longer than in *H. diversicolor aquatilis* in the present study. The factors controlling the increasing number of rows are not known, but this may be an interesting difference between the two abalone species.

Clearance angles of zero or less were suggested to indicate teeth would slide across the surface rather than cutting it (Padilla 1985). The curved radula teeth of postlarval *H. diversicolor aquatilis* <2 mm SL are probably suitable for collecting biofilm materials such as extracellular mucus secretions of diatoms, as suggested for postlarvae <1 mm of *H. iris* (Roberts et al. 1999b) and *H. discus hannai* (Kawamura et al. 2001). The increase in clearance angle in *H. discus hannai* of approximately 1 mm SL appeared to be related to the second transition in feeding, main diets being from mucous materials to tightly attached diatoms such as *Cocconeis* spp. colonizing on CCA (Kawamura et al. 2001). Postlarval *H. discus hannai* >1 mm SL with positive clearance angles of radula teeth seem able to detach *Cocconeis* cells from CCA surfaces, whereas smaller postlarvae with curved radula teeth cannot efficiently detach diatom cells (Kawamura et al. 2001). *H. diversicolor aquatilis* appears to efficiently detach strongly attached diatoms from CCA surfaces at 2–2.5 mm SL when the radula teeth have higher clearance angles. Biofilm components such as extracellular secretions from diatoms and/or from CCA can be important food sources for postlarval *H. diversicolor aquatilis* <2 mm SL, which is larger than in *H. discus hannai* (<1 mm SL). However, the size of food items that can be collected by the radula seems to increase with a reduction in tooth serrations (1 mm SL, Fig. 7) and an increase in the gap between the adjacent rows of teeth of postlarvae (>1.5 mm SL, Fig. 2). It should be noted here that the clearance angle at the point of contact with food items can change depending on various factors, such as the position of the radula when it protrudes for feeding, flexing of the radula during feeding, and the nature of the substrate. Our clearance angle data are based on the radulae removed from abalone and prepared for SEM. Therefore, the above discussion on the implications of feeding due to increased clearance angles is conjectural, as discussed previously in Roberts et al. (1999b) and Kawamura et al. (2001).

The L3–L5 teeth of juveniles and adults were sharply cusped without serrations and stood much higher above the radula membrane than rachidian and L1 and L2 teeth (Figs. 7D–F), suggesting that they are suitable for cutting firmly attached algae and/or for excavating substrata, as discussed for *H. iris* (Roberts et al. 1999b) and *H. discus hannai* (Kawamura et al. 2001). The differentiation of the L3–L5 teeth was considered to be related to the final transition in feeding of *H. discus hannai*, from microalgal to macroalgal feeding (Kawamura et al. 2001), though the marked increase in the activity of the macroalgal polysaccharide degrading enzymes observed at about 2 mm SL also appeared to contribute to the efficient utilization of macroalgae (Takami et al. 1998). In fact, postlarval *H. discus hannai* >1.8 mm SL used juvenile macroalgae *L. japonica* Areschoug 1851 efficiently (Takami 2002) after the

differentiation of lateral teeth, that started at ~1.5 mm SL (Kawamura et al. 2001). Although the adult complement of five pairs of laterals was completed at ~6 mm SL in *H. diversicolor aquatilis* (Fig. 6), the differentiation of the L3 teeth started at ~1.5 mm SL (Fig. 2) from the time they appeared. This suggests that *H. diversicolor aquatilis* also starts utilizing juvenile macroalgae in the late postlarval stage. Hence, *H. diversicolor aquatilis* may not need to use tightly attached diatoms before they start feeding on juvenile macroalgae, because they can feed and grow on biofilm mucus materials until ~2 mm SL, as discussed earlier.

Juveniles of *Haliotis discus hannai* and its subspecies *H. discus discus* initially feed on juvenile macroalgae (Maesako et al. 1984, Takami 2002) and/or soft algae such as *Ulva* spp. (Seki 1997). They began feeding on adult macroalgal fronds such as *Laminaria* and *Eisenia* once they became larger than ~13 mm SL (Seki 1997). The morphology of juvenile and adult radula teeth of *H. diversicolor aquatilis* is different from that of *H. discus hannai*. The sizes of L4 and L5 teeth of juvenile and adult *H. diversicolor aquatilis* were much smaller than L3 teeth (Figs. 7D–F, Fig. 8). Although we do not know how the abalone use different types of the radula teeth during feeding, the difference in radula morphology suggests that the main food items for juvenile and adult *H. diversicolor aquatilis* are different from *H. discus hannai*.

By comparing the radula morphology and its development with those of *H. discus hannai*, we can estimate the transitions in feeding of *H. diversicolor aquatilis* as earlier mentioned. Three possible differences in feeding and nutritional sources between the two species, which may be important from the ecologic point of view, can be considered: (1) The longer contribution of the residual yolk reserve as an energy source after metamorphosis is suggested for *H. diversicolor aquatilis*. This may mean longer starvation tolerance of newly metamorphosed postlarvae with limited and/or unsuitable food sources, or longer swimming larval life if they fail to contact an appropriate environmental stimulus for metamorphosis than those of *H. discus hannai* (Takami et al. 2000, Takami et al. 2002). (2) Because the differentiation of outer lateral teeth started almost at the same time of increasing clearance angles, main food items for *H. diversicolor aquatilis* can possibly transfer directly from biofilm mucus materials to juvenile macroalgae, and tightly attached diatoms may not be an essential food source in between them. Competition for tightly attached diatoms on CCA habitats between other herbivore gastropods was suggested to be a critical factor affecting the survival and growth rate of *H. discus hannai* (Takami et al. 2001, Kawamura et al. 2003). If the diatoms would not be necessary, the feeding and growth of *H. diversicolor aquatilis* could be more flexible than *H. discus hannai*. (3) The difference in the main food items for juveniles and adults between the two species, suggested in this study, may reflect differences in their actual habitats. The adults *H. diversicolor aquatilis* inhabit shallow boulder areas where large macroalgal forests are limited, whereas adult *H. discus hannai* are in deeper rocky areas with macroalgal forests. Further field observations and laboratory experiments are needed to clarify the feeding and nutritional sources of *H. diversicolor aquatilis* throughout their life stages.

ACKNOWLEDGMENTS

The authors thank Christopher B. Clarke for critical reading of a draft, and M. Hara for help with SEM observations.

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SEROTONERGIC AND FMRF-AMIDERGIC NEURONS IN THE NERVE GANGLIA OF *HALIOTIS ASININA* LINNAEUS

SASIPORN PANASOPHONKUL,¹ PRAPEE SRETARUGSA,^{1*} NARUMOL ANUNRUANG,¹
SONJAI APISAWETAKAN,¹ PORNCARN SAITONGDEE,¹ SUCHART E. UPATHUM,²
TANES POOMTONG,³ PETER J. HANNA,⁴ AND PRASERT SOBHON¹

¹Department of Anatomy, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand; ²Department of Medical Science, Faculty of Science, Burapha University, Chonburi 20130, Thailand; ³The Coastal Aquaculture Development Center, Department of Fisheries, Klongwan, Prachaubkirikhum Province 77000, Thailand; ⁴The School of Biological and Chemical Sciences, Deakin University, Geelong, Victoria 3174, Australia

ABSTRACT The neurons containing serotonin and FMRF-amide were immunohistochemically localized in the cerebral, pleuropedal and visceral ganglia of *Haliotis asinina*. The large ($\sim 10 \times 20 \mu\text{m}$ in diameter) and medium size ($\sim 7 \times 10 \mu\text{m}$ in diameter) neurons of these three ganglia contained immunoreactivities to both antibodies against serotonin and FMRF-amide, whereas the more numerous small size neurons ($< 6 \times 8 \mu\text{m}$ in diameter) did not show these immunoreactivities. The large neurons had oval-shaped nuclei containing mostly euchromatin and long cytoplasmic processes, whereas the medium size neurons, which could be neurosecretory cells, contained round nuclei with patches of heterochromatin and lack processes. The 5-HT immunoreactive cells were concentrated in the upper half of the medial edge of the cerebral ganglion, the edge of ventral and dorsal horns of the pleuropedal ganglion, and the right latero-ventral edge of the visceral ganglion; whereas the FMRF-amide immunoreactive neurons were concentrated in the dorsal and ventral edges of the cerebral ganglion, the edge of dorsal horn and the dorsal edge of the body of the pleuropedal ganglion, and the left latero-ventral and latero-dorsal edge of the visceral ganglion. The 5-HT cells were most numerous in the cerebral ($\sim 70/2,300$ cells per section), and the pleuropedal ganglia ($\sim 120/2,700$ cells per section), whereas they were least numerous in the visceral ganglion ($\sim 50/400$ cells per section). The FMRF-amide cells were most numerous in the cerebral ($\sim 76/2,000$ cells per section), and the pleuropedal ganglia ($\sim 130/2,500$ cells per section), whereas they were least numerous in the visceral ganglion ($\sim 60/1,050$ cells per section). The present study suggests that serotonin and FMRF-amide are major neurotransmitters in the neural ganglia, with only the large- and medium-sized neurons involved in the production and storage of these two neurotransmitters.

KEY WORDS: *Haliotis asinina*, serotonin, FMRF-amide, immunohistochemistry, nerve ganglia

INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) and tetrapeptide Phe-Met-Arg-Phe-amide (FMRF-amide) are neurotransmitters that play many important roles in controlling the physiologic and behavioral processes of invertebrates (Kuang et al. 2002, Mercier et al. 2003). In molluscs, 5-HT controls heartbeat (Skelton et al. 1992), locomotion (McClellan et al. 1994, Satterlie & Norekian 1995), feeding (Kupfermann & Weiss 1981), memory and learning (Nelson & Alkon 1997), and reproduction (Ram et al. 1996, Juneja & Koide 1996). FMRF-amide can evoke muscle tension (Schot & Boer 1982, Buckett et al. 1990), modulate cardiac output (Buckett et al. 1990), and reproduction (Lehman & Greenberg 1987). By immunolocalization methods, 5-HT and FMRF-amide have been observed in many ganglia of the central nervous system (CNS) of gastropod molluscs (Audesirk 1985, Elekes 1992, Croll et al. 2001, Fickbohm et al. 2001), especially in the pulmonate snails, *Helix pomatia* and *Helix aspersa* (Griffond et al. 1986, Lehman & Price 1987, Hernadi et al. 1989, Elekes & Nassel 1990, Hernadi 1992). Other gastropods that have also been investigated are a sea hare *Aplysia kurodai*, a land snail *Achatina fulica* (Fujii & Takeda 1988), an aquatic snail *Lymnaea stagnalis* (Audesirk 1985), and an opisthobranch *Phestilla sibogae* (Croll et al. 2001). The 5-HT and FMRF-amide immunoreactive neurons have been identified in the CNS of all these species with specific distribution patterns. For example, in *Aplysia kurodai* and *Achatina fulica*, the 5-HT-immunoreactive cells and neuropils were observed in all ganglia

except the left pleural ganglia. The sizes of these neurons are generally large, and some reach up to $50\text{--}70 \mu\text{m}$ in diameter. FMRF-amide immunoreactive neurons have been located throughout the central ganglia (cerebro-pleural, parietal, pedal, and visceral ganglia) of *Phestilla sibogae*, and about 65–100 intensely labeled neurons were observed in these ganglia, whereas another 40–45 neurons were weakly or variably stained (Croll et al. 2001). A similar study has been done in an aquatic snail, *Lymnaea stagnalis*, in which FMRF-amide immunoreactive neurons and neuropils were consistently found in the cerebral, pedal, right parietal, and visceral ganglia (Audesirk 1985). Moreover, the colocalization of 5-HT and FMRF-amide has also been performed in a chiton, *Lepidopleurus asellus*, which is a primitive mollusc species (Moroz et al. 1994). Both types of neurons are distributed in characteristic patterns in the CNS (i.e., the 5-HT immunoreactive neurons are concentrated in the ventral cords while FMRF-amide immunoreactive neurons in the lateral cords). In addition, a tight network of 5-HT and FMRF-amide immunoreactive nerve fibers were found in the body wall and the foot muscle (Moroz et al. 1994). The prevalence of serotonergic and FMRF-amidergic neurons in the CNS in gastropods implies that they are important neurotransmitters in these animals.

In contrast to the higher gastropods, very few works along this line have been carried out in the primitive gastropods. Only two studies reported the presence of 5-HT in the cerebral ganglion of developing larvae of red abalone, *Haliotis rufescens* (Kataoka et al. 1987, Barlow & Truman 1992) and 5-HT-immunoreactive fibers in the outer zone of the retinal plexiform layer and the optic nerve trunk (Kataoka et al. 1987). In the present study, we have examined and compared the distribution of 5-HT and FMRF-

*Corresponding author: E-mail: scpsr@mahidol.ac.th

amidergic neurons in the neural ganglia of the CNS of *Haliotis asinina*, a tropical abalone commonly found in Thai coastal waters.

MATERIALS AND METHODS

Tissue Collection

Adult male *H. asinina* (age >16 mo) were collected from a land-based aqua culture system at Coastal Aquaculture Develop-

ment Center, Department of Fisheries, Prachaukirikhun Province, Thailand. They were kept in concrete tanks housed in the shade and well flushed with mechanically circulated sand-filtered seawater and provided with an air delivery system to maintain the stable controlled environment. The optimum level of salinity is about 22.5–32.5 ppt and temperature $\sim 22^{\circ}\text{C}$ to 26°C (Singhagravan & Doi 1999). They were fed with macroalgae (usually *Gracilaria* spp. and *Laminaria* spp.), supplemented with artificial food.

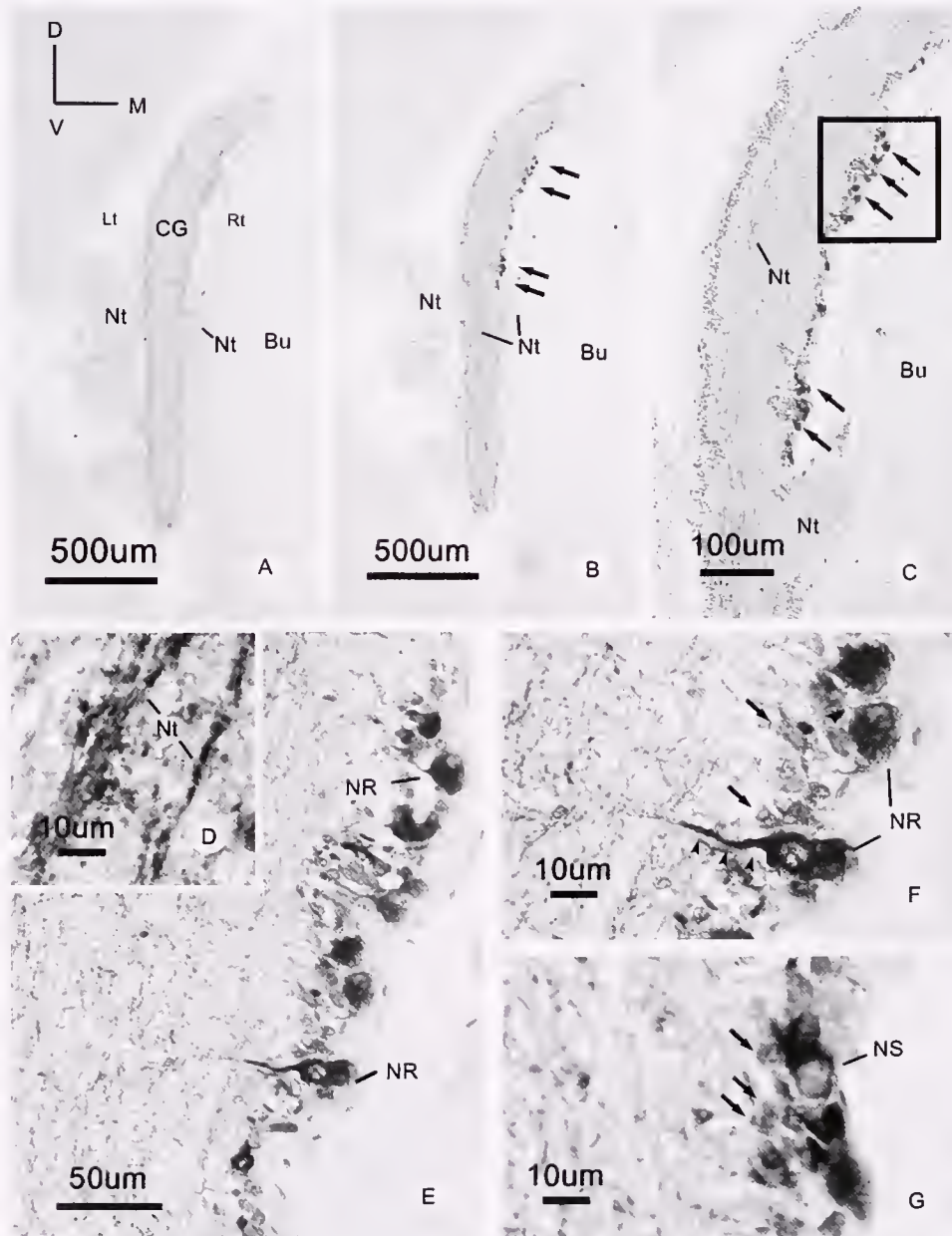


Figure 1. LM micrographs illustrating the location and characteristics of serotonergic neurons and nerve fibers in the cerebral ganglia of *H. asinina*: A. A negative control cross section showing a cerebral ganglion (CG) and nerve bundles (Nt) arising from the ganglion to buccal mass (Bu). D = dorsal, V = ventral, M = medial, Lt = left side, Rt = right side. B & C. 5-HT neurons (black dots- arrows), concentrated in the upper half of the cortex at the medial edge of the ganglion, with the immunoreactive nerve fibers (Nt) distributed throughout the neuropil of the ganglion, and the nerve branch running into the buccal mass (Bu). D. A high-power micrograph of the neuropil of a cerebral ganglion, showing the varicosities of 5-HT immunoreactive nerve fibers (Nt). E. A micrograph from the boxed area in C, showing a row of serotonergic neurons in the cortex of the ganglion. F & G. High-power micrographs of the two types of serotonergic neurons from E, the large neuron (NR) characterized by the presence of long axon (arrowheads), and the medium-size neurosecretory neuron (NS). No immunoreactivity is detected in the small neurons (arrows).

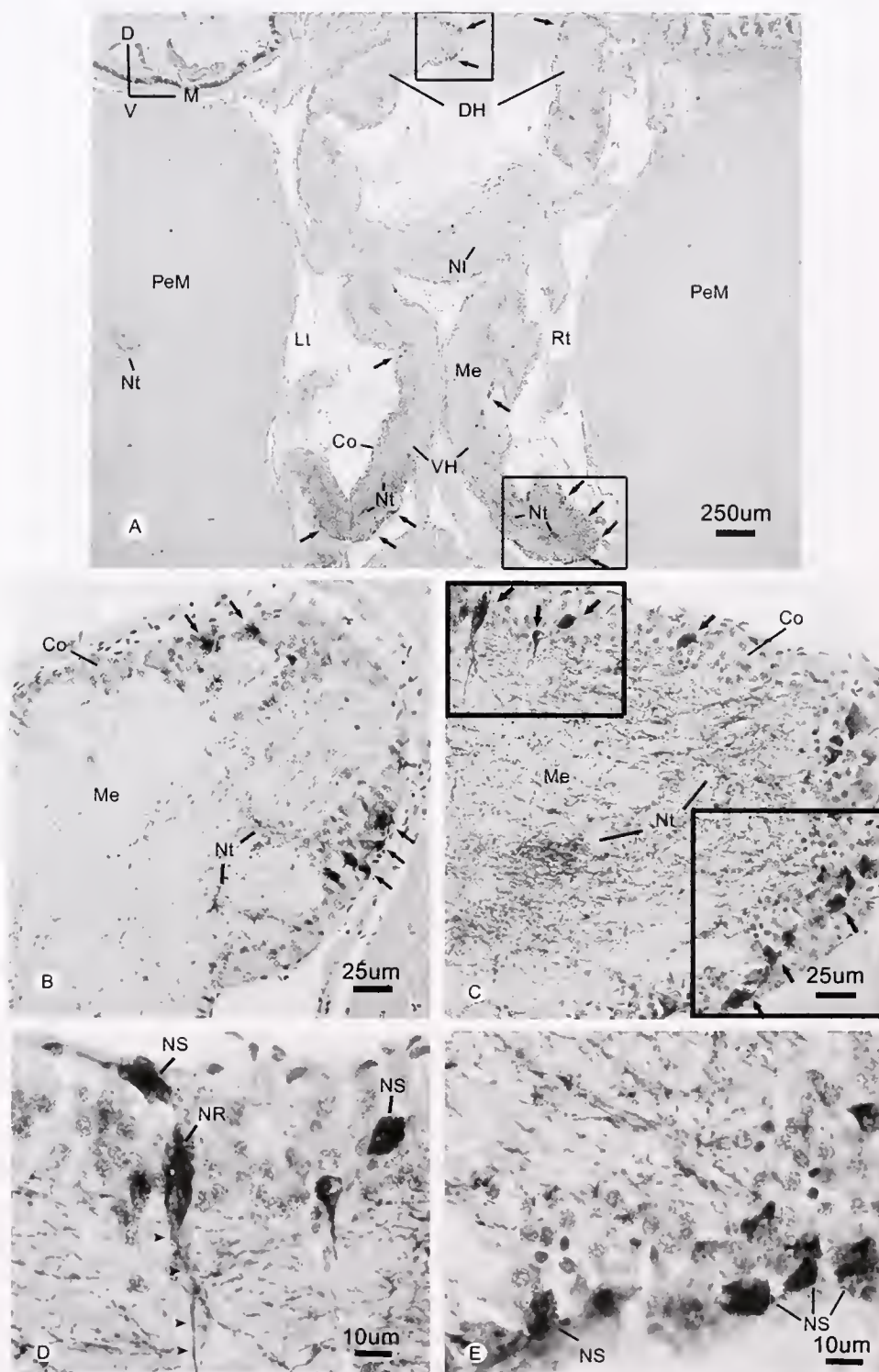


Figure 2. LM micrographs of a mid cross section of the pleuropedal ganglia of *H. asinina*, illustrating the distribution and characteristics of serotonergic neurons and nerve fibers. A. A low-power micrograph, showing the distribution of 5-HT neurons (arrows) concentrated in the upper or lower one-third of the cortex (Co) of the dorsal (DH) or ventral horns (VH). The positively stained nerve fibers (Nt) are also present in the medulla (Me) and pedal muscle (PeM). Lt = left, Rt = right. B & C. Medium-power micrographs of the boxed areas of left dorsal horn (B) and right ventral horn (C) in A, showing groups of serotonergic neurons (arrows) in the cortex (Co) and positively stained nerve fibers (Nt) in the medulla (Me). D & E. High-power micrographs of serotonergic neurons from the boxed area in C, showing the large size neuron (NR) characterized by the long axon (arrowheads), and medium-size neurosecretory cells (NS) without processes.

Antibodies Against 5-HT and FMRF-Amide

The rabbit polyclonal antibody against 5-HT was purchased from Zymed Laboratories Inc. For antibody against FMRF-amide, the peptide (Sigma Company) was dissolved in deionized water at

0.5 mg/mL. This solution was mixed with keyhole limpet hemocyanin (KLH) (10 mg/mL) in 1:1 ratio. Then, glutaraldehyde was added up to 0.375% with 1 M glycine-HCl. After that, the solution was mixed with 0.1 M phosphate buffered saline (PBS) containing complete Freund's adjuvant in 1:1 ratio for subcutane-

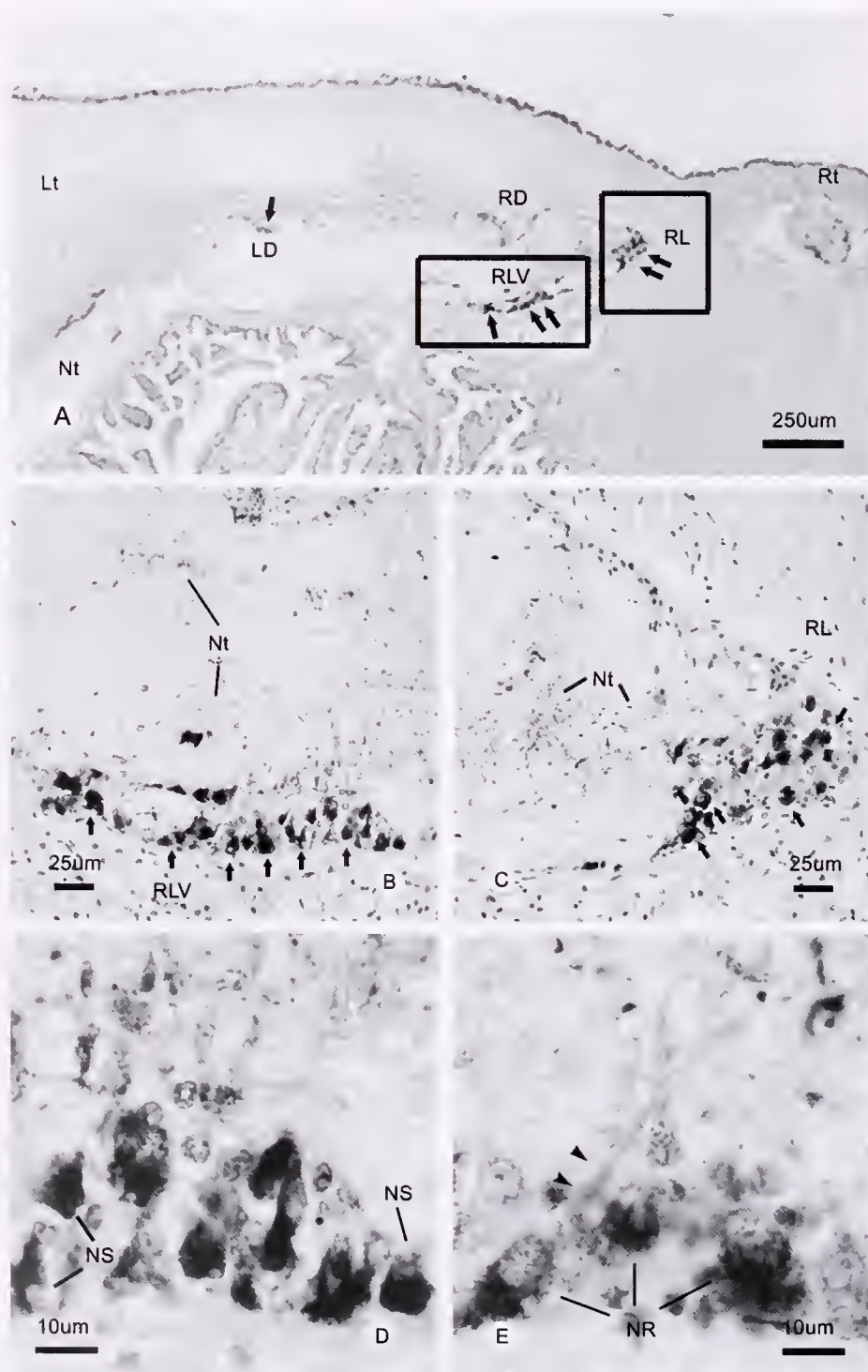


Figure 3. LM micrographs of a cross section of a visceral ganglion, illustrating the location and characteristics of serotonergic neurons and nerve fibers. A. A low-power micrograph, showing two groups of serotonergic neurons (arrows) in the right lateral (RL-arrows) and right latero-ventral (RLV-arrows) edge of the ganglion, whereas only few positive cells are found in the right and left dorsal edge (RD, LD-arrows). A few immunoreactive nerve fibers (Nt) are also present in the ganglion. B & C. Medium-power micrographs, showing groups of 5-HT neurons from the boxed areas in A, and the 5-HT immunoreactive nerve fibers in the medulla region. D & E. High-power micrographs, showing both types of large (NR) and medium (NS) size serotonergic neurons in the boxed areas in A.

ous injection into experimental mice (300 μ L/mouse). After 14 days, a similar dose of FMRF-KLH mixed with incomplete Freund's adjuvant was injected to boost the immune response of the animals. On day 49, the blood was collected from the heart bleeding and centrifuged at 5,000g, and the serum was collected, mixed with 20 folds in volume of KLH solution to preabsorb antiKLH in the antiserum. Then, the dot blot ELISA was used to detect the specificity of the mouse antiFMRF-amide polyclonal antibody, and make sure that antiKLH was completely absorbed. The pre-absorbed antiserum was used to stain the sections of cerebral, pleuropedal and visceral ganglia.

Immunoperoxidase Method

Ten adult male *H. asinina* weighing more than 20 g were anesthetized with 5% $MgCl_2$, then the shells were removed. Cerebral, pleuropedal, and visceral ganglia were dissected out and fixed in Bouin's solution at 4°C for 12–16 h. Specimens were washed in 70% ethanol for removal of the fixative. Then, they were dehydrated through a graded series of ethanol (70% to 100%) for 30–45 min each depending on the size of the specimens, cleared with dioxane, infiltrated and embedded in paraffin wax, and sectioned at 5- μ m thick. For immunostaining, the paraffin

sections of each ganglion were deparaffinized with xylene, rehydrated through a graded series of ethanol (100% to 80%), and finally in 70% ethanol containing 1% Lithium carbonate ($LiCO_3$). The endogenous peroxidase was blocked by treating the sections with 3% H_2O_2 in methanol for 30 min. Then, the sections were covered with 4% bovine serum albumin (BSA) in 100 mM phosphate buffered saline, containing 0.25% triton-X 100, pH 7.4 (PBST), for 30 min. Following the blocking step, the consecutive sections were incubated in either the rabbit anti5-HT or mouse antiFMRF-amide serum as the primary antibodies, for 1 h, at 37°C. After incubation, the sections were washed three times with PBST, then incubated in the HRP-conjugated goat antirabbit or anti mouse IgG as the secondary antibodies, for 30 min, at 37°C. Finally, the sections were immersed in the substrate solution containing 0.03% w/v DAB, 0.3% $NiCl_3$, and 0.1% H_2O_2 in 50 mM Tris buffer pH 7.2, washed several times with distilled water, counter-stained with Hematoxylin, and mounted in the permount solution. The sections were observed and photographed by a Nikon microscope equipped with digital camera DXM 1200. In addition, the numbers of serotonergic and FMRF-amidergic neurons in each ganglion of each abalone were counted and estimated as number of positive cells per total number of cells per section, which were taken at the middle of each ganglion.

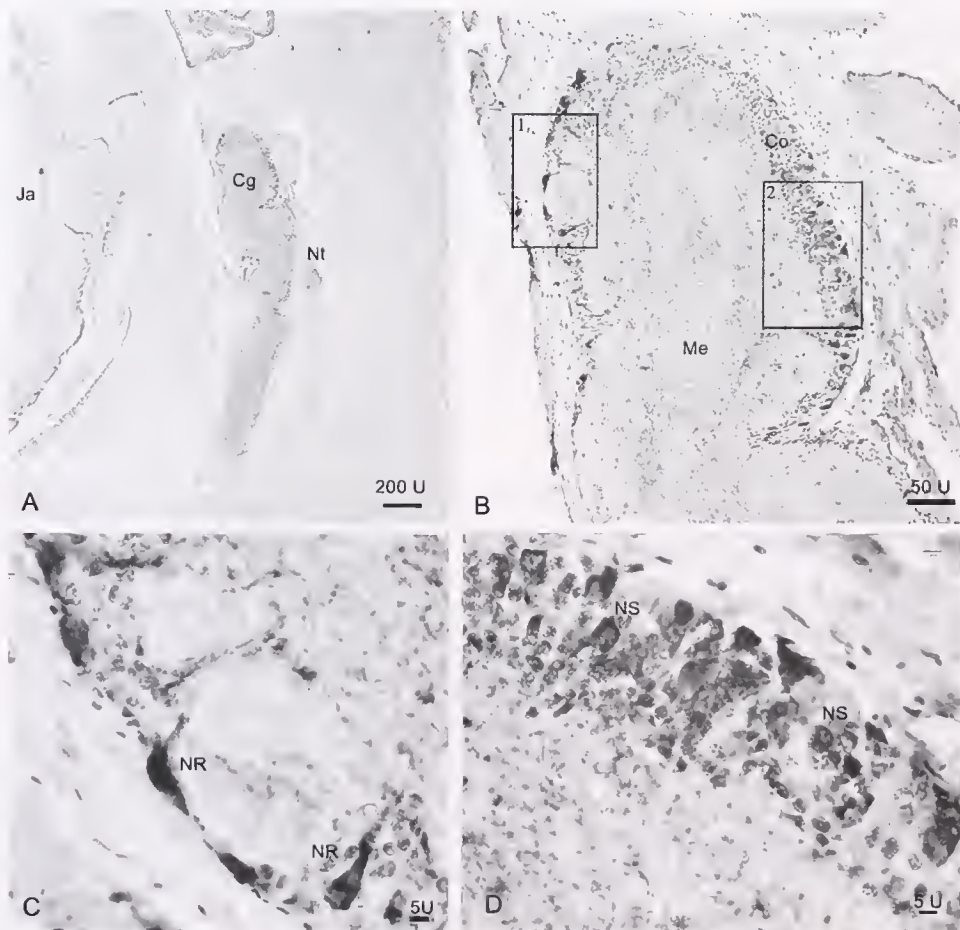


Figure 4. A. Low-power micrograph of a cross section of cerebral ganglia showing the distribution of the FMRF-amide cells (black color) concentrated at the upper and lower parts of lateral and the dorso-medial edges of the ganglion. B. Medium-power micrograph, showing the concentration of immunoreactive FMRF-amide cells in the cortex (Co) of the upper half of the cerebral ganglion, and numerous immunoreactive nerve fibers in the medulla (Me). C & D. High magnification showing the FMRF-amide immunoreactive large neurons (NR) and medium size neurons (NS) in the cortex of the cerebral ganglion. Ja = jaw.

RESULTS

Immunohistochemical Localization

By using immunoperoxidase technique enhanced with nickel chloride (NiCl_2) we were able to localize two separate sets of neurons containing 5-HT and FMRF-amide in the cerebral, pleuropedal and visceral ganglia. The patterns of distribution of these two types of neurons in each ganglion are described below.

Cerebral Ganglion

The 5-HT cells are concentrated in the upper half of the cortex of medial edge of the ganglion (Figs. 1B, C, Fig. 7A), whereas there are few widely scattered 5-HT cells in the lateral edge of the ganglion. FMRF-amide cells are concentrated in the cortex of the dorsal and ventral edges of the ganglion whereas the lateral edge and the dorso-medial edge have only few widely scattered FMRF-amide cells (see Figs. 3A, B, Fig. 7A). Both 5-HT and FMRF-amide immunoreactive nerve fibers were also observed throughout the neuropils of the ganglia. The 5-HT immunoreactive nerves branching out from the ganglia were also observed around the head

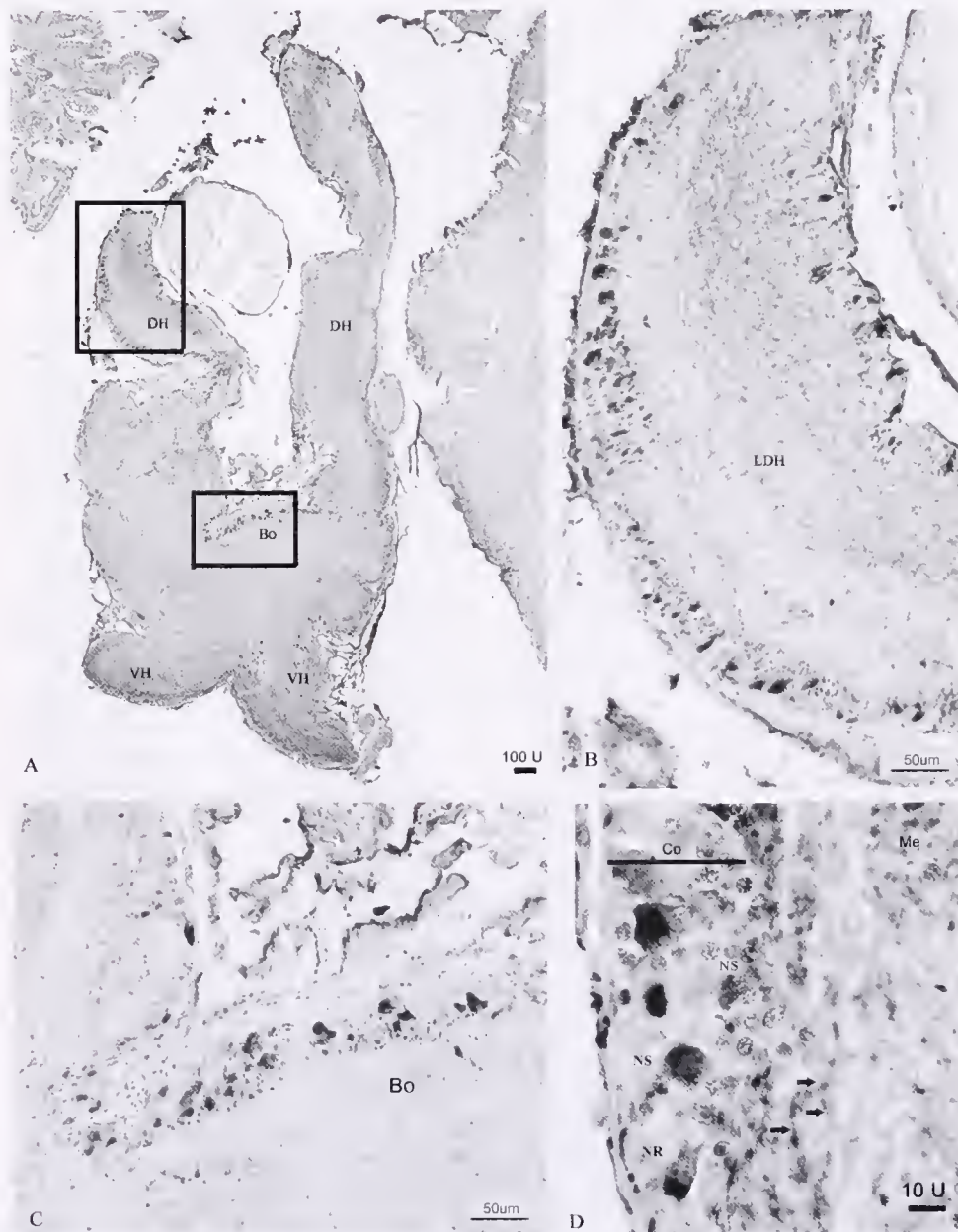


Figure 5. A. Low-power micrograph showing the distribution of the FMRF-amide cells (black color) concentrated in the cortex of the upper one-third of both medial and lateral edges of the dorsal horn (DH), and the dorsal edge of the body part (Bo) of the pleuropedal ganglia. B & C. Medium-power micrographs of FMRF-amide immunoreactive cells in the cortex of left dorsal horn (LDH) and dorsal edge of the body (Bo) of pleuropedal ganglia. D. A high-power micrograph of left dorsal horn (DH) in A, showing immunoreactive large (NR) and medium size neurons (NS) in the cortex (Co), and nerve fibers with varicosities (arrows) in medulla (Me) of the ganglia.

and buccal areas of the abalone, but fewer FMRF-amide immunoreactive fibers were found (Fig. 1B, see Fig. 4A later). Based on the histologic characteristics, the serotonergic neurons could be classified into 2 types (i.e., the large and medium size neurons). The large oval or pyramidal shape neurons (about $10 \times 20 \mu\text{m}$) contain the oval-shaped nuclei with complete euchromatin, and usually large nerve processes (Figs. 1E, F), whereas the medium size neurons (about $7 \times 10 \mu\text{m}$), which could be neurosecretory cells, contain round nuclei with patches of heterochromatin (Upatham et al. 1998, Kruatrachue et al. 1999, Thongkukiatkul et al. 2000) and lacking processes (Fig. 1G). However, there was no immunoreactivity of 5-HT in the cell bodies of small neurons ($<6 \times 8 \mu\text{m}$) (Figs. 1F, G). Similarly, from the histologic characteristics, FMRF-amidergic neurons are also mostly the large size neurons and medium size neurons, with no staining in the small neurons (Figs. 4C, D). The number of 5-HT cells are about

70/2,300 cells per section in comparison to 76/2,000 cells per section for FMRF-amide cells.

Pleuropedal Ganglion

In pleuropedal ganglia, the 5-HT cells were found concentrated in the upper and lower one-third part of the dorsal and ventral horns of the ganglion (Fig. 2A to C, Fig. 7B) with widely scattered 5-HT cells on the lateral edge of the ventral horn (Fig. 2A, Fig. 7B). FMRF-amide cells were found concentrated in the cortex of the upper one-third of the dorsal horn and the dorsal edge cortex of the body, with few widely scattered positive cells in the ventral horn and the ventral edge of the body (Fig. 5A). The immunoreactivity of both types of nerve fibers were also found throughout the neuropils of the ganglia, especially in the dorsal and ventral horn regions, (Fig. 5A, C). Histologically, there are only two types

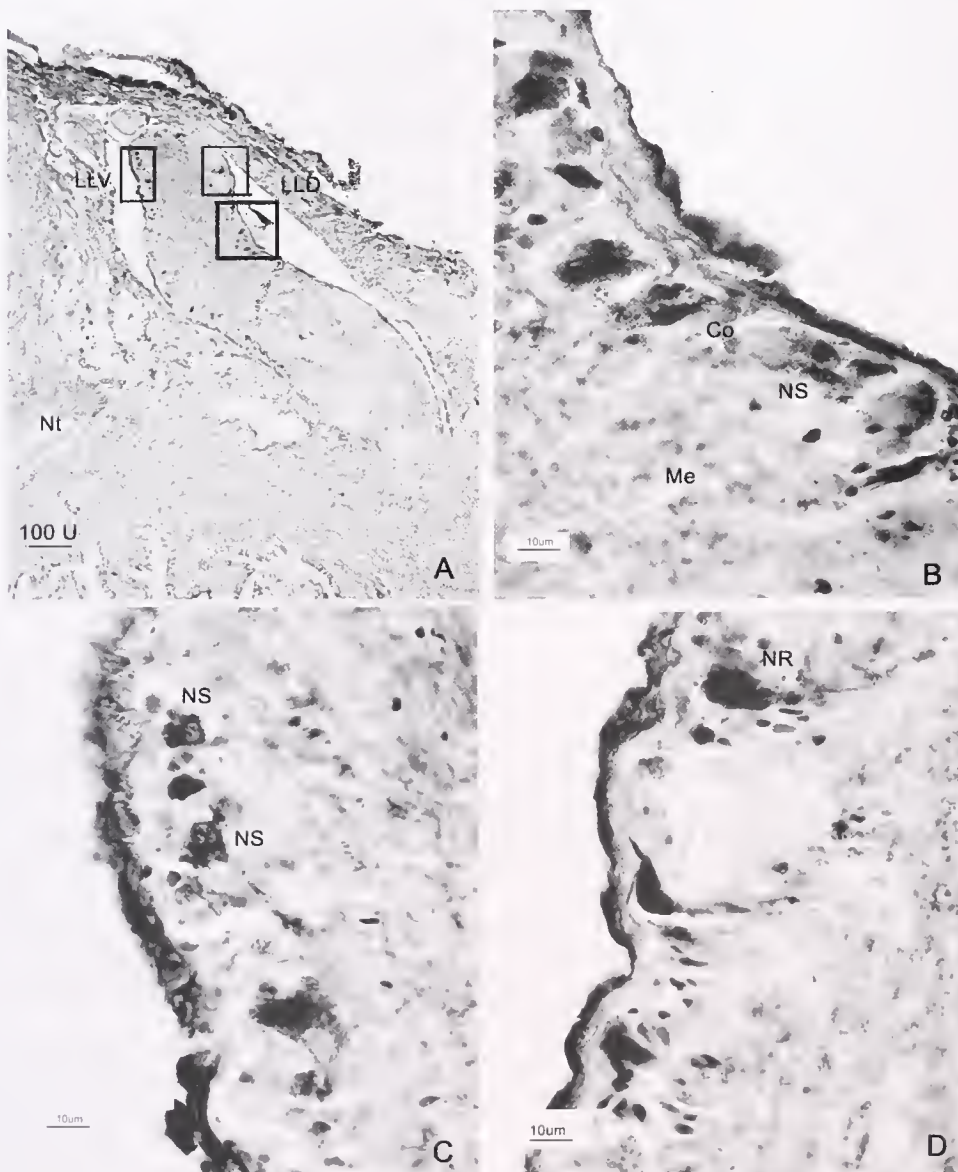


Figure 6. A. A medium-power micrograph and high-power micrographs (B, C, & D) of the visceral ganglion, showing the immunoreactive large (NR) and medium size neurons (NS) in the cortex region (Co) of the left latero-ventral (LLV) and dorsal (LLD) edges of the ganglion.

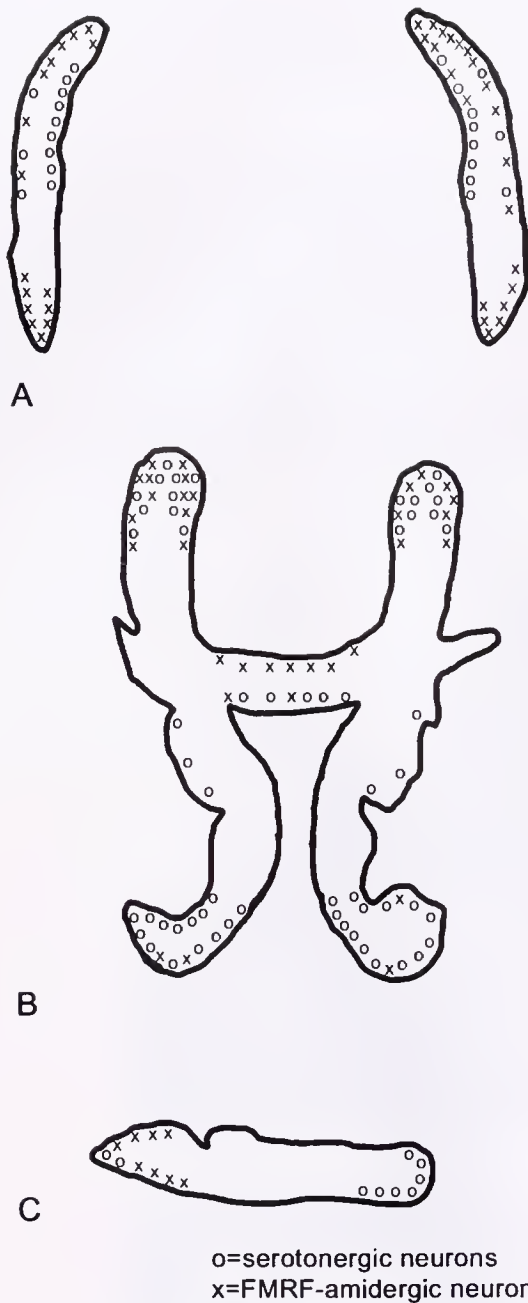


Figure 7. A schematic diagram summarizing the distinctive distribution patterns of 5-HT and FMRF-amide immunoreactive neurons in the cerebral A, pleuropedal B, and visceral ganglia C of adult *H. asinina*. O = serotonergic neurons, x = FMRF-amidergic neurons.

of immunoreactive 5-HT and FMRF-amide neurons with similar characteristic as those found in the cerebral ganglia (Figs. 2D, E, Fig. 5D). The numbers of both types of these neurons are approximately equal and they are evenly distributed in specific areas of the ganglia as mentioned. There are about 120/2,700 cells per section for serotonergic neurons in the ganglion, and about 130/2,500 cells per section for FMRF-amide cells.

Visceral Ganglion

The 5-HT neurons are concentrated on the right lateral and latero-ventral edge of the ganglion (Figs. 3A to C, Figure 7C),

while the FMRF-amidergic neurons are concentrated on the left latero-ventral and latero-dorsal edge (Fig. 6A, Fig. 7C). A few immunoreactive nerve fibers containing 5-HT and FMRF-amide were also observed in the neuropil of the ganglion (Figs. 3A to C). The two types of immunoreactive neurons for both 5-HT and FMRF-amide could also be identified, with similar characteristics as in the cerebral and pleuropedal ganglia (Figs. 3D, E, Fig. 6B to D). The number of serotonergic neurons is about 50/400 cells per section, whereas that of FMRF-amide cells is about 60/1,050 cells per section.

DISCUSSION

The distribution of 5-HT and FMRF-amide, the major neurotransmitters, have been widely studied in a number of invertebrates (Beltz & Kravitz 1983, Nassel et al. 1985, Fujii & Takeda 1988, Too & Croll 1995, Raikova et al. 2000) including the gastropod molluscs (Audesirk 1985, Elekes 1992, Croll et al. 2001, Fickbohm et al. 2001). However, most previous studies have been done in the opisthobranch and pulmonate species, the higher gastropods. Up to now, there are few works on the prosobranch including abalone, which belong to the most primitive subclass of gastropods (Barlow & Truman 1992).

In the present study, we have studied the distribution patterns of these two types of neurotransmitters in the central ganglia of *H. asinina*. The results showed the presence of immunoreactive 5-HT and FMRF-amide neurons in all three ganglia, with the distinctive distribution patterns in each ganglion as summarized in Figure 7. Both serotonergic and FMRF-amidergic neurons also showed a tendency to be grouped into clusters as in higher gastropods, whereas in species of the lower classes; such as, platyhelminthes, annelida, and arthropoda, serotonergic neurons tend to be more widely scattered (Fujii & Takeda 1988).

H. asinina possesses a fairly high number of both serotonergic and FMRF-amidergic neurons in all three ganglia, especially in the pleuropedal ganglia. Fujii and Takeda (1988) found that the number of serotonergic neurons in invertebrates tends to increase in the higher phyla. Furthermore, it was found that one of the main factors that controls the number of 5-HT cells is the physiologic condition. In a snail *Helix pomatia*, it was reported that the number of serotonergic cells in the central ganglia changed with the physiologic conditions of the animals (i.e., they increased during active phase and decreased during hibernation) (Hiripi & Salanki 1973).

While the immunoreactive 5-HT and FMRF-amide neurons were observed in all three ganglia, the immunoreactive nerve fibers containing 5-HT were found concentrated in the neuropils of the ganglia, as well as in musculature of the head, buccal, and foot regions, whereas FMRF-amide fibers were only found in the neuropils and around the hemolymph sacs. These characteristics were similar to the higher gastropod molluscs (Schot & Boer 1982, Fickbohm et al. 2001). Therefore, it is possible that serotonergic neurons may play a primary role in controlling the muscle contraction for head and foot movement and feeding, as in other gastropods, whereas FMRF-amidergic neurons may function mainly as a neuromodulator in the CNS.

The sizes of both serotonergic and FMRF-amidergic neurons are different from those in other gastropods, especially in a pulmonate and opisthobranch molluscs, also in which the positive neurons showed remarkable size range (Fickbohm et al. 2001). In *H. asinina*, both immunoreactive neurons are confined to the large nerve cells with processes and the medium size nerve cells with no processes. The large neurons could be equivalent to the large neu-

rons as classified by Kruatrachue et al. (1999), which seem to be the motor neurons controlling the musculature of head, buccal, and foot region via their long axon. The medium size cells have all characteristics of neurosecretory cells (Kruatrachue et al. 1999). Interestingly all small size nerve cells, which are believed to be association or interneurons, are nonimmunoreactive. Thus, the strong presence of 5-HT and FMRF-amide in the CNS of *H. asinina* suggests that both neurotransmitters have important roles in

controlling the physiologic and behavioral responses of this animal as in the other gastropods.

ACKNOWLEDGMENTS

This research was supported financially by the Thailand Research Fund (Senior Research Scholar Fellowship to Prasert Sobhon, and the Royal Golden Jubilee Ph.D. Scholarship to Sasiporn Panasophonkul).

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SENSORY RECEPTORS ON CEPHALIC AND EPIPODIAL TENTACLES OF *HALIOTIS ASININA* LINNAEUS

CHAITIP WANICHANON,^{1,*} PRAPHAPORN LAIMEK,¹ NATPILA CHITCHULANON,¹
WORAWIT SUPHAMUNGMEE,¹ SOMJAI APISAWETAKAN,¹ VICHAI LINTHONG,¹
PRAPEE SRETARUGSA,¹ MALEEYA KRUATRACHUE,² EDWARD SUCHART UPATHAM,³
TANES POOMTONG,⁴ AND PRASERT SOBHON¹

Departments of ¹Anatomy and ²Biology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand, ³Department of Medical Science, Faculty of Science, Burapha University, Chonburi 20130, Thailand, ⁴The Coastal Aquaculture Development Center, Department of Fisheries, Klongwan, Prachuabkirikun Province 77000, Thailand

ABSTRACT *Haliotis asinina*, a tropical abalone, has a pair each of cephalic, optic, appendage tentacles at the anterior end of the head, and numerous epipodial tentacles distributed on the periphery of its body. The cephalic and epipodial tentacles are essentially sensory organs with similar general structure. In a mature adult (about 16 mo-old), the cephalic tentacle measures about 3.77 cm in length and 0.14 cm in diameter, whereas the epipodial tentacle measures about 9.27 mm in length and 0.59 mm in diameter. In cross sections, each tentacle has a bundle of nerve fiber forming the core structure, surrounded by a thick layer of circular muscle, which also branches into radially oriented fibers. These fibers are interlaced with thick paraxially orientated fibers; together they account for the size and mobility of the tentacles. The surface of both types of tentacles can be divided into three parts: the basal part exhibits slight corrugation consisting of small folds alternated with grooves, the middle part has numerous short hillock-shaped papillae, and the top part has a very high concentration of cone-shaped papillae. Each papilla comprises a group of densely stained ciliated neuroepithelial cells, surrounded by lightly stained supporting epithelial cells bearing microvilli. Gamma amino butyric acid (GABA) was found, by immunohistochemistry, to be highly concentrated in the neuroepithelial cells. The rest of epithelium of both kinds of tentacles is of a columnar type, consisting of clear cells bearing microvilli and goblet cells. The GABA-containing neuroepithelial cells are scattered widely among them.

KEY WORDS: *H. asinina*, tentacle, histology, sensory receptor, GABA

INTRODUCTION

Tentacles are important sense organs of gastropods, which contain tactile and chemoreceptor cells in abundance (Chase 1981, Chase & Croll 1981). The cephalic tentacles are among the most important prosobranch sense organs, although little attention has been paid to their histologic structure and function. They are richly endowed with sensory cells, which may be significantly different in each of the major groups of gastropods (Haszprunar 1988, Beesley et al. 1998). Ito et al. (2000) stained the neurons in the tentacles in a terrestrial slug, *Limax marginatus*, by backfilling of the tentacular nerves with Lucifer yellow. Four types of stained neurons comprising sensory neurons, gamma cells, ganglion cells, and lateral cells, were identified in the superior and inferior tentacles. In *Haliotis tuberculata*, a temperate species of abalone, the cephalic tentacle has a mixed sensory and motor nerve, which are centrally located and extends along the length of the tentacle. On the periphery of the nerve, there are sinuses located between muscle fibers, and the latter are arranged longitudinally, obliquely and transversely. Covering the muscle is the surface epithelium that is shown to have folds or papillae (Crofts 1929). The epithelium is of cuboidal type, which consists of 3 cell types: sensory cells, supportive epithelial cells, and widely scattered mucus cells. The sensory cells are spindle-shaped with darkly stained nuclei and supporting cells that are oval and more transparent (Crofts 1929, Bevelander 1988). The epipodium is a series of small tentacles arising from the dorsal part of the foot. They often have the same shape and structure as the cephalic tentacles, although they are much smaller (Crofts 1929, Haszprunar 1988). Crofts (1929) in-

dicated that the epipodial tentacles of *H. tuberculata* had 2 types of epithelial cells: supporting cells and sensory cells.

The present study reports on the structure of both the cephalic and epipodial tentacles of *Haliotis asinina* Linnaeus, a tropical abalone commonly distributed along the Thai coastal water, as observed by light and scanning electron microscopes. In addition, immunohistochemistry showed that GABA, a major neurotransmitter of gastropods, is highly concentrated in the sensory epithelial cells.

MATERIALS AND METHODS

Collection of Abalone Specimens

Adult abalones over 12-mo-old were obtained from a land-based culture system at Coastal Aquaculture Development Center, Department of Fisheries, Prachuabkirikun Province, Thailand. They were reared in concrete tanks housed in the shade, and well flushed with mechanically circulated sand-filtered seawater, and provided with an air delivery system to maintain a stable controlled environment. The optimum level of salinity used was ~22.5–32.5 ppt, and the temperature about 22 °C to 26 °C (Singhagraiwan & Doi 1993). They were fed with macroalgae (usually *Gracilaria* spp. and *Laminaria* spp.), supplemented with artificial food.

They were anesthetized in 5% MgCl₂, after which their shells were removed. The tentacles (cephalic, epipodial and appendage) were measured, dissected out and processed for light microscope (LM) and scanning electron microscope (SEM) studies.

Specimen Preparation for SEM

Tentacles were cut and fixed in a Karnovsky fixative (4% glutaraldehyde 2% paraformaldehyde in 0.1M sodium cacodylate

*Corresponding author. E-mail: scewn@mahidol.ac.th.

buffer), pH 7.8, at 4 °C overnight, and washed in 0.1 M sodium cacodylate buffer. They were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h, at 4 °C. Then, they were dehydrated in graded series of ethanol, and dried in a Hitachi HCP-2 critical point drying machine, using liquid CO₂ as a transitional medium. They were then mounted on aluminum stubs and coated with platinum and palladium in an ion sputtering apparatus, Hitachi E 5000. The specimens were examined in a Hitachi S-2500 scanning electron microscope with an accelerating voltage of 15 kV.

Specimen Preparation for LM

Specimens were fixed in Bouin solution in 0.14 M NaCl for 24 h and washed with 70% ethyl alcohol. They were then dehydrated through a graded series of ethanol, cleared with dioxane, infiltrated and embedded in paraffin. Five-micron-thick sections were cut and stained with Harris hematoxylin and eosin.

For semithin section, specimens were fixed in Karnovsky fixative, pH 7.8, at 4 °C for overnight, and washed with 0.1M sodium cacodylate buffer. They were postfixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer, at 4 °C, for 1 h. Then, they were dehydrated in a graded series of ethanol and embedded in Araldite 502 resin. Sections were cut at 1-μm thickness with Porter Blum MT-2 μL tramicrotome, and stained with methylene blue or PAS-methylene blue. Examination and photographing of the tissue sections were done under a Nikon eclipse E600 microscope and Nikon digital camera DXM1200.

Immunoblotting for GABA

The neural and tentacular tissues were tested for the presence of GABA by homogenizing the cerebral ganglia, pleuropedal ganglia, tentacular, and epipodial tentacles collected from adult *H. asinina* in 0.1 M sodium phosphate buffer saline (PBS) containing 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF). The homogenate was centrifuged at 12000g for minutes, after which the supernatant was collected, and the protein contents determined by Lowry method (Lowry et al. 1951). Proteins were adjusted to be about 1 mg/mL by adding the extraction buffer. A 1-μL of each suspension at the dilutions at 1:1, 1:10, and 1:100 were spotted onto nitocellulose (NC) sheets. The NC sheets were then incubated in 5% skimmed milk for blocking of nonspecific binding before being incubated in the primary antibody (mouse monoclonal antibody against GABA, Sigma Chemical Co. USA). Peroxidase-conjugated goat antimouse IgG (Zymed Laboratories) was used as the secondary antibody. The antigen-antibody complexes were then visualized by enhanced chemiluminescence using a LumiGlo kit (KPL, Gaithersburg, MA), with detection made on X-ray film. For positive control neural tissues, the mouse brain was collected and treated similarly.

Immunohistochemistry

Additional 5-μm sections of paraffin-embedded tentacular and epipodial tentacles, as prepared for LM study, were placed on glass slides, deparaffinized, and rehydrated in ethanol and distilled water. They were then stained by an immunoperoxidase method, by first immersing in 0.1M PBS, pH 7.4, containing 0.3% H₂O₂ to block endogenous peroxidase, followed by 0.1% glycine in buffer to block free aldehyde groups from the fixative, then in 10% normal goat serum in the buffer for 1 h, in monoclonal antibody (MoAb) (Sigma Chemical Co. USA) at 1:300 dilution, at 20 °C overnight. The sections were thoroughly washed with PBS, followed by incubation in HRP-conjugated secondary antibody (Goat antimouse IgG-HRP, Zymed Laboratories) at 1:200 dilution, for 2 h, and labeled with an aminoethyl carbazole substrate kit (AEC, Zymed Laboratories) for 30 min. Finally, the sections were counter-stained with Harris hematoxylin before being mounted in buffered glycerol, and photographed in Nikon eclipse E600 microscope and Nikon digital camera DXM1200.

RESULTS

Cephalic Tentacle

Cephalic tentacles of mature female abalone (age about 16 mo) were 3.77 ± 0.59 cm in length and 0.14 ± 0.03 cm in diameter (Table 1); whereas the length of cephalic tentacles and the diameters of mature male abalone of similar age was 3.38 ± 0.57 cm and 0.12 ± 0.02 cm, respectively. Noticeably, at the same age the female shell was longer than that of male, and its weight was also heavier than that of male (Table 1).

A cephalic tentacle is round and tapered from the base to the tip (Figs. 1A to C). Based on the surface features as observed under SEM, the tentacle could be divided into 3 parts (i.e., 1/10 basal, 1/10 middle and 8/10 top). There are gradual changes between adjacent parts. The basal part has a smooth surface. It consists of many folds, alternated with grooves (Fig. 1E). Many sensory papillae are located on the folds. On the middle part of the tentacle, there are more papillae on each fold (see Fig. 3C). These papillae are distributed separately and appear taller than those at the base. Each appears as a hillock covered with microvilli and encircling cilia at the tip (Figs. 2C, E). On the top part of the tentacle, the papillae are longer and more numerous than those on the middle part (Fig. 1F). Each appears as a slender truncated cone, projecting perpendicularly to the tentacle's surface (Figs. 2A, F). On top of each papilla, there is a tuft of long cilia, whereas the rest of its surface is covered with microvilli (Figs. 2D,F).

In paraffin sections, the longitudinal section of a cephalic tentacle also exhibits the three parts, based on the degree of surface folding that show gradual changes between adjacent parts (Fig.

TABLE 1.
Sizes of cephalic tentacles and epipodial tentacles of adult *H. asinina*.

Sex	Weight (g)	Shell Length (cm)	±SD	Cephalic Tentacle Length (cm)	±SD	Diameter of Cephalic Tentacle (cm)	±SD	Epipodial Tentacle Length (cm)	±SD	Diameter of Epipodial Tentacle (cm)	±SD
Male	29	3.77	0.62	3.38	0.57	0.12	0.02	0.80	0.24	0.06	0.01
Female	33	4.11	0.64	3.77	0.59	0.14	0.03	0.93	0.32	0.06	0.02

All data are based on 16-mo-old fresh abalone of each sex ($n = 15$). SD, standard deviation.

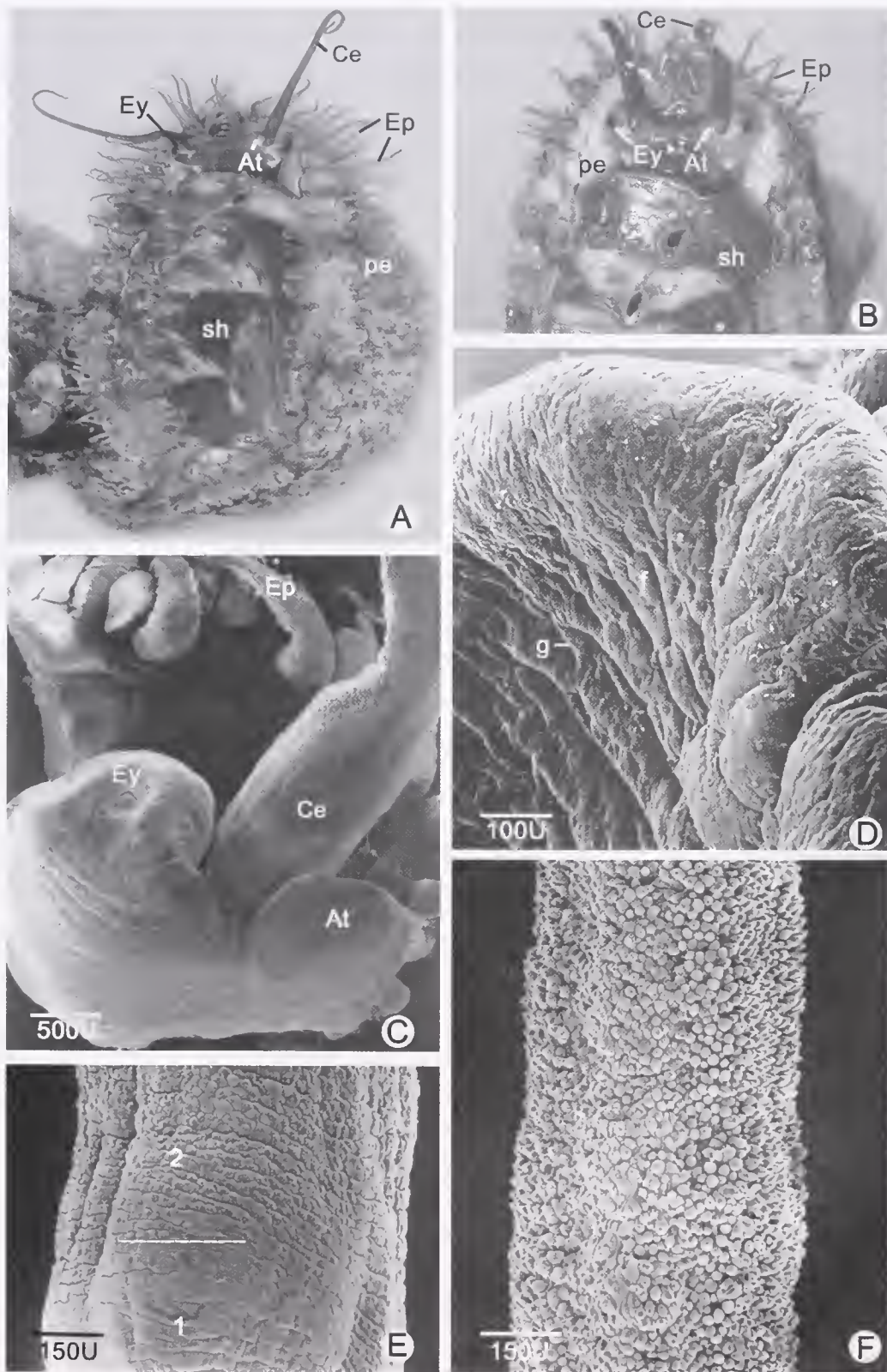


Figure 1. A & B. SEM photographs of the dorsal view showing the external features of an adult (16 mo old) *H. asinina*. At = appendage tentacle, Ep = epipodial tentacle, Ey = eye, pe = pedal muscle, sh = shell, Ce = cephalic tentacle. C. A low-power SEM micrograph showing the location and appearances of the optic (Ey), cephalic (Ce), and appendage (At) tentacles from lateral to medial. D. Higher magnification of the appendage tentacle showing many grooves (g) and folds (f). E. A low-power SEM micrograph of a cephalic tentacle shows the basal (1) and middle (2) parts of the tentacle. F. A low-power SEM micrograph of the cephalic tentacle shows the top part of the tentacle that has numerous sensory papillae.

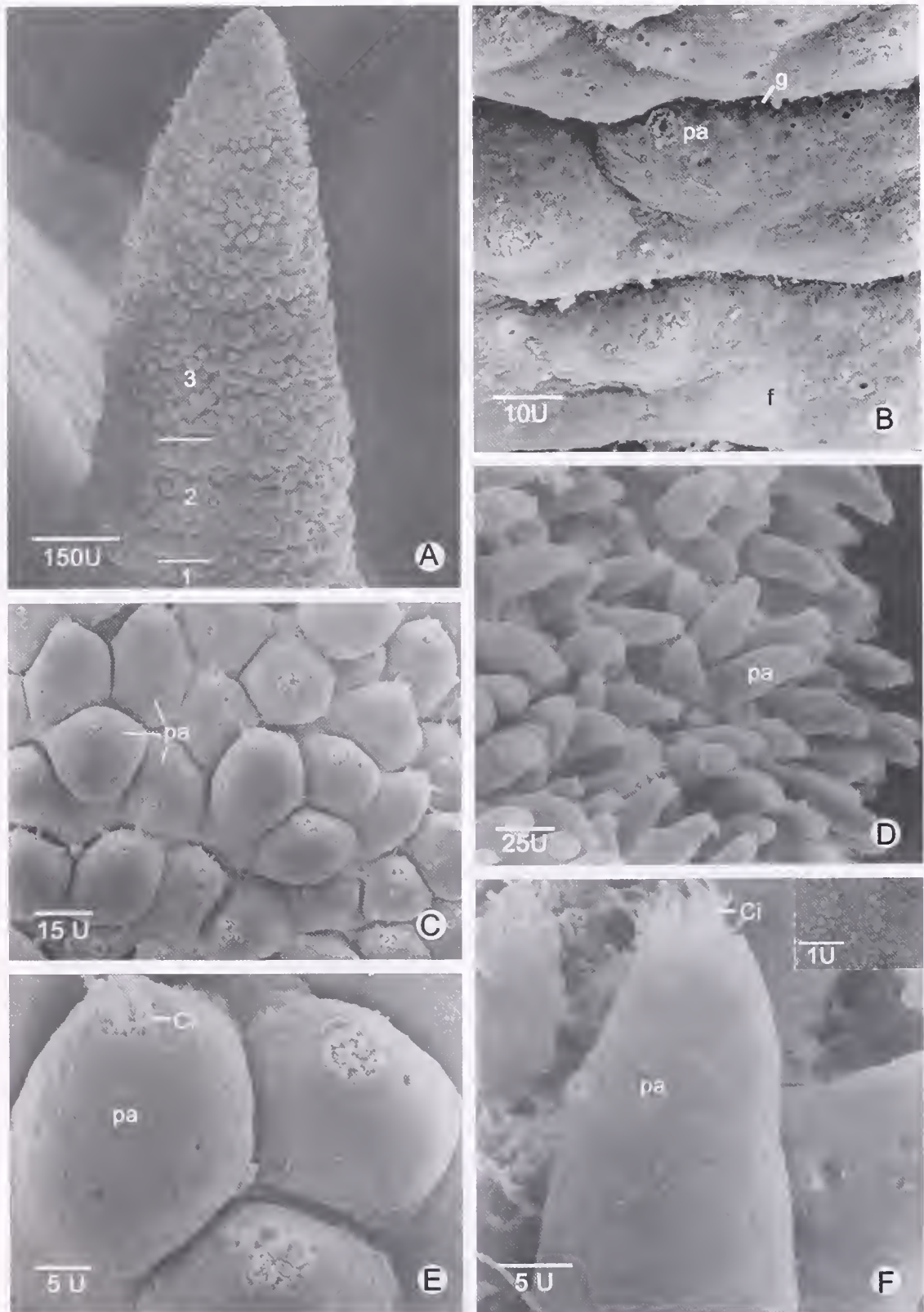


Figure 2. A. A low-power SEM micrograph showing the cone-shaped and knobby surface of an epipodial tentacle that is divided into 3 areas: basal (1), middle (2), top (3) parts. B. A medium-power SEM micrograph of the basal part showing grooves (g) alternated folds (f); the former contain many short papilla (pa). C. A medium-power SEM micrograph of the middle part of cephalic tentacle showing the surface that has short but stout papillae (pa). D. A medium-power SEM micrograph of the papillae (pa) on the top part of a cephalic tentacle, all of which are long and have a cone shape. E. A high-power SEM micrograph of a papilla of the middle part showing a bulbous shape with a tuft of cilia (Ci) on top. F. A high-power SEM micrograph of the top part showing many tall cone-shaped papillae (pa) with numerous long cilia (Ci) on top, whereas the rest of its surface is covered with microvilli (inset).

3A). Along the axis of each tentacle, there is a central tentacular nerve running along its whole length, and gives rise to the radial branches that innervate the surrounding muscle (Figs. 3B, C). Covering the tentacle is a simple columnar epithelium lying on a thick basement membrane (Figs. 3D, E). The cells in the epithelium covering the papilla can be classified into three types, based on their histologic characteristics and staining affinities. They are: (1) a sensory cell that is spindle shaped with a round or oval shape nucleus, and contains darkly-stained chromatin (Figs. 3D, E). The cytoplasm is dense and stained blue with methylene blue and pinkish purple with PAS-methylene blue. There are long cilia at the tip of each sensory cell, which give rise to the ciliary tuft at the top of each papilla as observed under SEM; (2) a supporting cell which has a columnar or pyramidal shape, and it is generally larger than the sensory cells. It has a round or oval nucleus that contains mostly euchromatin with a few blocks of heterochromatin (Figs. 3D, E). The cytoplasm is clearer than sensory cell, and appears light blue after staining with methylene blue, and light pinkish with PAS-methylene blue stain. The supporting cell has a brush border that is made of microvilli; and (3) a goblet cell, which is a type of mucin-producing cell with a small nucleus and oval shape that is mostly euchromatin (Fig. 3D). The cytoplasm is large, clearer, and stained light blue with methylene blue and pinkish with PAS-methylene blue. At the point where sensory cells aggregate to form a papilla, fibers from the tentacular nerve terminate at a group of these cells that have cilia on top (Fig. 3E).

Epipodial Tentacle

The general structure of an epipodial tentacle is similar to that already described for a cephalic tentacle, but each tentacle is about two times narrower and four times shorter than the cephalic tentacle (Fig. 1A, Table 1). Based on the surface features observed under SEM, each tentacle could also be divided into 3 parts (i.e., 1/5 basal, 1/5 middle, and 3/5 top) (Fig. 2A). Each part has characteristic structures (Figs. 2B, C, D), but there are gradual changes between adjacent parts. The surface of the basal part has folds alternated with grooves (Fig. 2B). On the folds are many short bulbous papillae, each has a circle of cilia on the top (Fig. 2B). In contrast, at the middle part the grooves and folds become less obvious, whereas the papillae are wider and longer, and each appears as a hillock with a circle of cilia on top (Figs. 2C, E). On the top part of an epipodial tentacle there are numerous papillae that are more slender than those on the lower parts, and each appears as a truncated cone with a circle of cilia at the top (Figs. 2D, F).

In spite of its much shorter length, the epipodial tentacle histologically resembles the cephalic tentacle. There are a bundle of epipodial tentacle nerves in the central axis (Figs. 4A, B). Most of surface epithelium, particularly at the basal part of the tentacle, is covered by columnar cells bearing a brush border (Figs. 4C, D). The papilla comprises of three types of epithelial cells with the same characteristics and staining affinities as those of papillae located on a cephalic tentacle (Figs. 4C, D).

Appendage Tentacle

In addition to the cephalic, optic, and epipodial tentacles, *H. asinina* has a pair of vestigial appendage tentacles. Each is short

with a half circle shape, and is covered by numerous irregular folds, alternated with grooves (Fig. 1D). The folds are covered by epithelial cells bearing numerous microvilli.

Transverse sections of the appendage tentacle reveal similar interior structure (i.e., nerve and muscle) to that of the cephalic and epipodial tentacles. The columnar epithelium, which covers the tentacle, also comprises three types of cells that are evenly spread over the surface without being organized into sensory papillae.

Immunoblots of GABA

The extracts of *H. asinina* neural tissues (i.e., cerebral and pleuropedal ganglion) are strongly stained for GABA almost equal to that of mouse brain extract (Fig. 5). However, both tentacular and epipodial tentacular tissues are only slightly stained for GABA.

Sensory Cells Containing GABA

Both of the cephalic and epipodial tentacles have similar patterns of immunostaining for GABA in the cells of epithelium and in the muscle (Fig. 6B). The most prominent immunoreactive cells were sensory cells aggregated at the center of each papilla, which could be readily identified by the presence of cilia on their apical surface (Fig. 6C). Of the remaining surface epithelial cells, the immunoreactive cells are sensory cells, which are widely scattered among the supporting cells (Figs. 6D to F). The nerve bundle and its radiating nerve fibers in both types of tentacles exhibited GABA immunoreactivity (Fig. 6B). In the control the immunostaining is negative (Fig. 6A).

DISCUSSION

Cephalic and Epipodial Tentacles

As in other prosobranch mollusks, the cephalic tentacles of *H. asinina* are the major sensory organs for perceiving the environment conditions because they are the outgrowths from the anterior end of the head (Zaitseva 1997, Beesley et al. 1998). In cross sections the basic structure of the cephalic tentacle is similar to that of other species of *Haliotis* because the tentacular nerve is in the middle of longitudinally orientated muscle fibers, and it is covered by a simple columnar epithelium (Croft 1929, Bevelander 1988, Beesley et al. 1998). In this study, it was found that the epithelium of cephalic tentacles of *H. asinina* has 3 types of cells: sensory cells, supporting cells; which are the majority of cell type, and mucus cells. This is in agreement with the study by Croft (1929) who reported similar finding in the cephalic tentacle of *H. tuberculata*. The sensory cells of the tentacles of *Haliotis* are not sub-epithelial as in pulmonate mollusks (Wright 1974), but are part of the epithelium similar to that in *P. elegans* (Zaitseva 1997). The sensory cells could be easily identified as having oval shaped nucleus with densely stained chromatin and cytoplasm in contrast to the supporting cells that have much clearer nuclei and cytoplasm. In the basal part of the cephalic tentacle, they are widely scattered in the epithelium and are located in the middle and top parts and concentrated in the center of each papilla. Underneath the papilla, there is a nerve branch from the central tentacular nerve bundle that may supply the sensory cells. This situation is similar to the nerve bundles of *P. elegans*, which connect their sensory dendrites to the epithelium of the tentacle tip (Zaitseva 1997). On

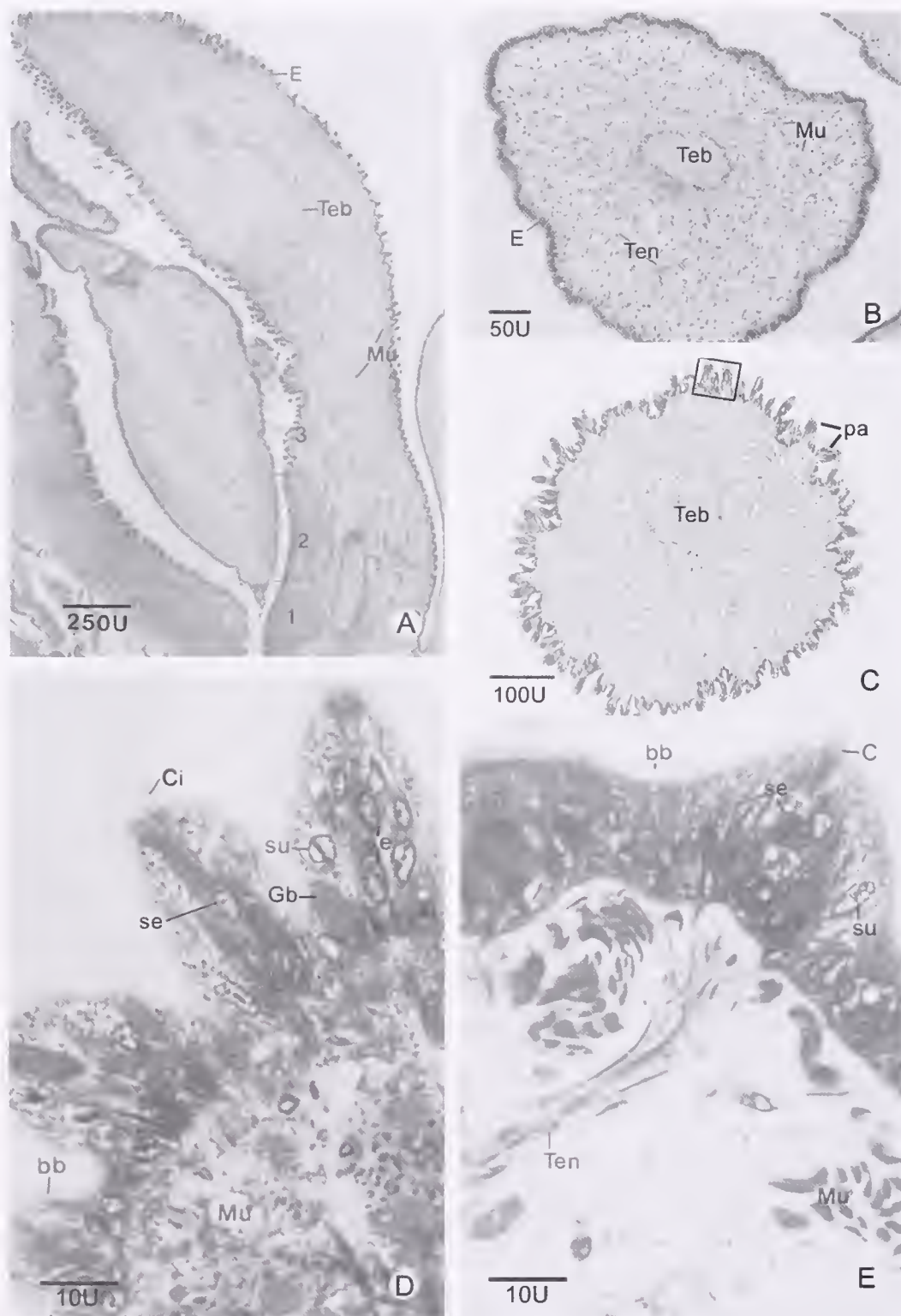


Figure 3. Paraffin sections (A to C) and semithin sections (D & E) of cephalic tentacles. A. A low-power LM micrograph of a longitudinal section of cephalic tentacle showing 3 parts: basal (1), middle (2) and top (3). Note the presence of cephalic tentacular nerve bundle (Teb) in the axis of the tentacle. Mu-muscle fasciculus. B & C. A medium-power LM micrograph of a cross section of the basal and top parts, respectively, showing cephalic tentacular nerve bundles (Teb) in the axis. The tentacle is surrounded by an epithelium. E. Mu-muscle fasciculus, pa-papillae, Ten-tentacular nerve. D. A high-power LM micrograph of a semithin plastic section of the top part stained with methylene blue, showing 3 types of cells in the papillae: sensory cell (se), supporting cell (su) and goblet cell (Gb). A group of cilia (Ci) is present on the top of each papillae and linked with the sensory cells, the papillae are more slender and longer than those on the middle part of the tentacle. E. A high-power LM micrograph of a semithin plastic section of the basal part stained with methylene blue, showing an epithelium with brush border (bb). The epithelium contains sensory cell (se), supporting cell (su). Muscle fasciculi (Mu) are seen in the connective tissue underlying the epithelium.

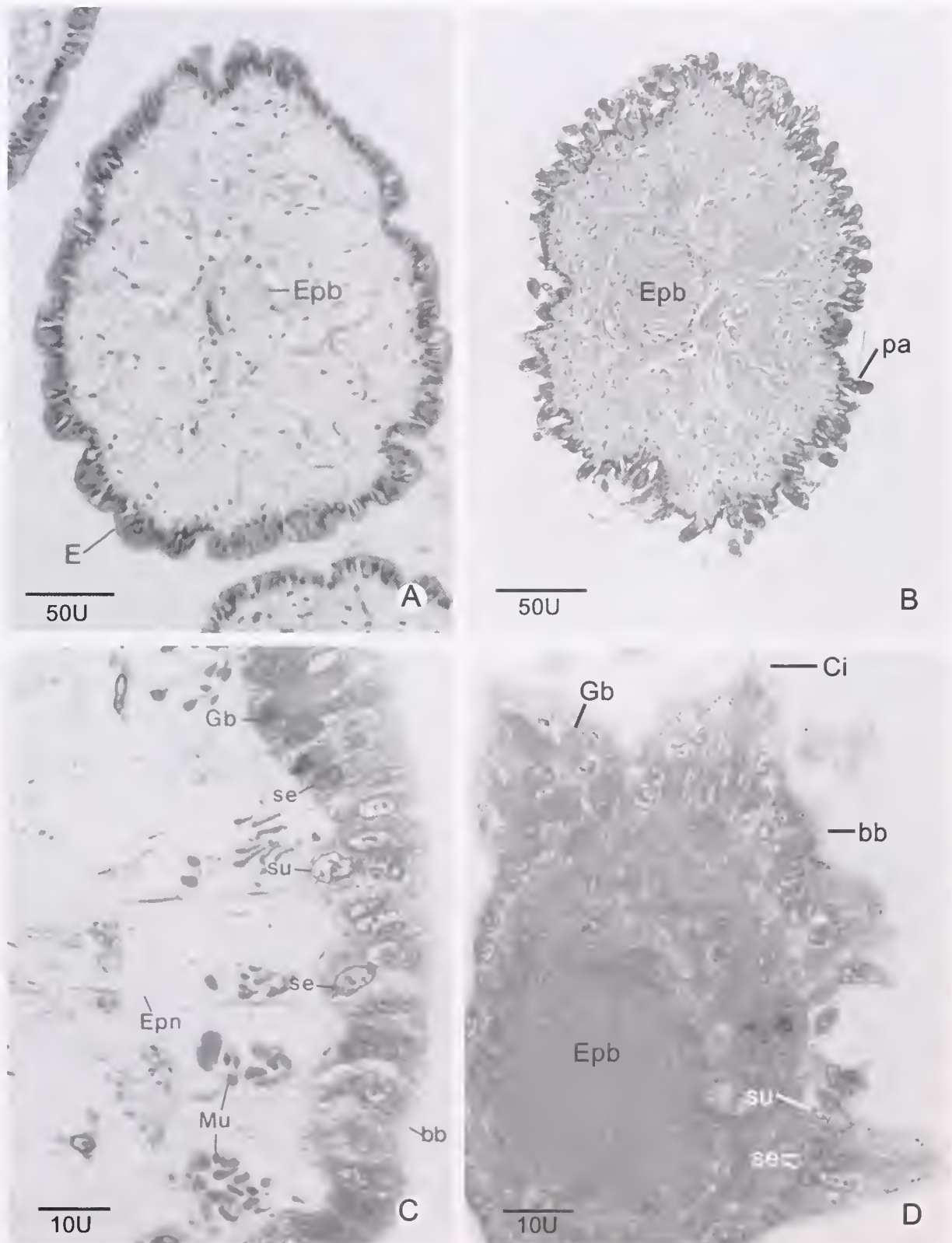


Figure 4. Paraffin sections (A & B) and semithin sections (C & D) of epipodial tentacles. A. A low-power LM micrograph of a cross section of the basal part showing surface epithelium (E) that has few papillae, and a nerve bundle (Epb) in the axis. B. A low-power LM micrograph of a cross section of the top part showing numerous surface papillae (pa) and a nerve bundle (Epb) in the axis. C. A high-power micrograph of interpapillary area of the basal part of an epipodial tentacle stained with methylene blue, showing surface epithelium with brush border (bb), but no papillae. The epithelium consists of goblet cell (Gb), sensory cell (se), and supporting cell (su). The underlying connective tissue contains muscle fasciculus (Mu) and nerve (Epn) to innervate the sensory cells of the epithelium. D. A high-power LM micrograph of the papillae, showing sensory cell (se) with cilia (Ci) at the center of a papilla, supporting cell (su) and goblet cell (Gb). The epithelium is covered with brush border (bb).

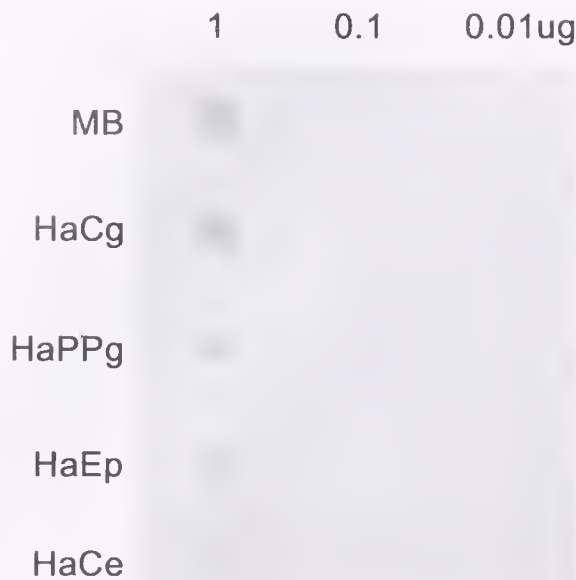


Figure 5. Chemiluminescent detection of GABA in immunoblots of abalone tissues. Extracts of tissues from cephalic tentacle (HaCe), epipodial tentacle (HaEp), cerebral ganglion (HaCg), and pleuropedal ganglion (HaPPg) were spotted in duplicate onto a nitocellulose membrane at 0.01, 0.1, and 1 µg of protein in the extraction buffer. All extracts were positively stained for GABA as well as mouse brain (MB), used as a positive control.

the apical surface of each papilla, SEM showed that there was a bundle of long twisted cilia that in cross section were demonstrated to belong to the sensory cells. In contrast, the supporting cells bear only microvilli that appear in LM as a brush border. The goblet cells are also widely scattered in the epithelium, and few are located in the papilla epithelium. Unlike the epithelium lining the mantle and the hypobranchial gland, the tentacular epithelium is probably not the major site for mucin production because it specializes mostly in the sensory perception.

The epipodial tentacles are smaller and shorter than the cephalic tentacles. They are numerous and located around the foot muscle. They have been shown in this study, as well as in the study by Crofts (1929), to have a basic structure similar to that of the cephalic tentacles. However, the muscle bundles in the epipodial tentacles are smaller and do not appear as regularly arranged as those in the cephalic tentacles. The cephalic tentacles are used for exploring the environment and for seeking out food, while avoiding danger; therefore, they need longer and stronger muscle bundles to stretch and retract them over a long distance. Alternatively the epipodial tentacles function more in support of the cephalic tentacles in finding food and receiving chemical stimuli around the abalone's body. Hence, epipodial tentacles are smaller and shorter than the cephalic tentacles and only project out for a short distance around the body, therefore these tentacles do not need as strong a muscle as in the former.

The group of ciliated sensory cells in the papillae of cephalic and epipodial tentacles are structurally very much like those in the olfactory epithelium or taste buds of vertebrates (Kierszenbaum 2002). Thus, the papillae may function mostly as chemoreceptors similar to the olfactory epithelium and taste buds of the vertebrates. This is supported by many investigators who have shown

that, in addition to tactile reception, the cephalic tentacles also act as chemical receptors because they can respond to the chemical stimuli, odor, and food (Preston & Lee 1973, Farkas & Shurey 1976, Chase 1981, Bell & Tubin 1982, Voss & Schmidt 2001). Shimozono et al. (2001) reported that the metacerebro-procerebral neuron (MPN) of *Limax marginatus* is an output-neuron from the procerebrum. The MPN receives monosynaptic inputs from the superior and inferior tentacle nerves. The MPN, thus, may receive olfactory information via two pathways (i.e., one directly from the tentacle and the others by way of the procerebrum) and possibly functions by integrating both sources of inputs. Similar sensory inputs may be gathered by epipodial tentacles because their papillae have identical structural organization.

Immunohistochemistry

This study has demonstrated the presence of GABA cells of both cephalic and epipodial tentacles. Identification of the immunostained cells is based on the characteristics and the position of cells. These cells are mostly the sensory cells aggregated at the center of each papilla, or single sensory cells widely scattered in the rest of the tentacular epithelium. GABA is the major inhibitory neurotransmitter in the central nervous system and a neuromodulator in certain peripheral tissues vertebrates (Sattelle 1992). In marine invertebrates, GABA is a neurotransmitter that stimulates growth and metamorphosis of the larvae into more mature stage (Aminur & Veharai 2001), and in abalone it specifically stimulates settlement and metamorphosis (Morse et al. 1988) and feeding of larvae (Arshavsky et al. 1993). Therefore, it is highly possible that the GABAergic sensory cells in the papillae of both cephalic and epipodial tentacle play important parts in sensing the availability of food and perhaps also controlling the feeding behavior of the postmetamorphosed abalone up to and during the adult stage.

Appendage Tentacle

The pulmonate mollusks have 2 pairs of tentacles: the superior and inferior tentacles, and the anterior and posterior tentacles, which are called the cephalic tentacle and rhinophore (Beesley et al. 1998). Rhinophores have shapes ranging from simple tapering rods to elaborate lamellae or tubular organs (Beesley et al. 1998). In the prosobranch mollusks, there is one pair of cephalic tentacles, and they do not have a rhinophore (Beesley et al. 1998). In this study, we found a pair of short and small tentacles that had a dome shape and were located dorso-medial to the base of the cephalic tentacle. They were called appendage tentacles. It is possible that these tentacles are homologous to the pulmonates rhinophores, but they remain as vestigial structures. The appendage tentacle has a simple columnar epithelium containing supporting and mucus cells but much fewer sensory cells; there were no papilla like those on the cephalic and epipodial tentacles, and there are no well organized muscles and nerve fibers. Thus, they may just be rudimentary structures.

ACKNOWLEDGMENTS

This research was supported financially by the Thailand Research Fund to Prasert Sobhon for a Senior Research Scholar Fellowship.

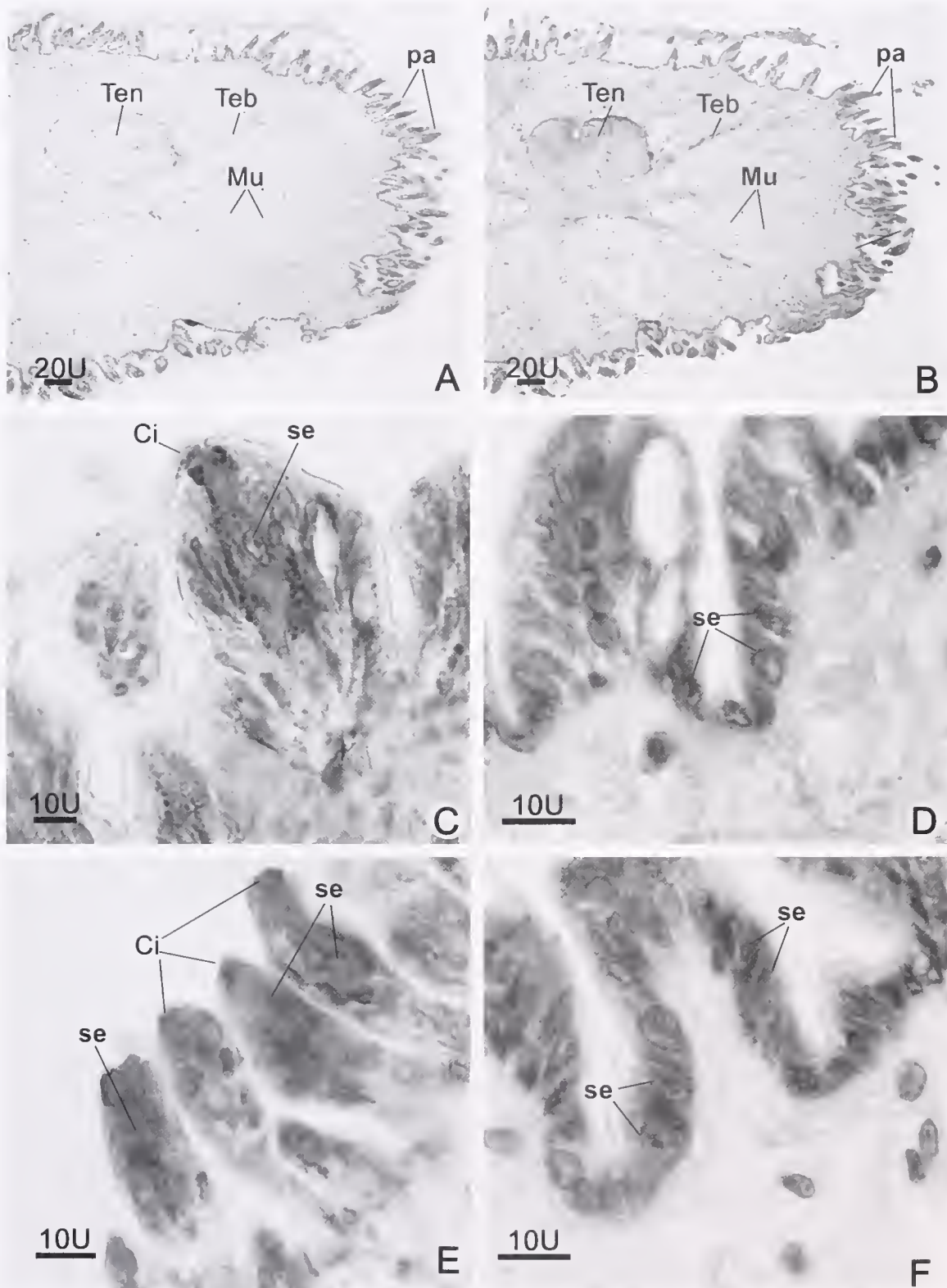


Figure 6. Light micrographs of the cephalic and epipodial tentacle sections stained for GABA by an immunoperoxidase method. A. Control section shows no staining in the cross section of a cephalic tentacle. B. An adjacent paraffin section stain with antiGABA showing positive staining in the epithelium covering papillae (pa) and the nerve bundle (Teb) and its fibers (Ten) traversing the muscle fasciculus (Mu). C & D. Higher magnifications of sections of the epithelium covering cephalic tentacles stained for GABA, and showing intense staining of the sensory cells (se) at the tip and the base of a papilla. E & F. A cross section of the upper part of an epipodial tentacle epithelium stained for GABA, and showing the same pattern of positive staining in the sensory cells (se) at the tip and the base of the papillae.

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HISTOLOGY OF HYPOBRANCHIAL GLAND AND GILL OF *HALIOTIS ASININA* LINNAEUS

CHAITIP WANICHANON,^{1,*} PRAPHAPORN LAIMEK,¹ VICHAI LINTHONG,¹
PRAPEE SRETARUGSA,¹ MALEEYA KRUATRACHUE,² EDWARD SUCHART UPATHAM,³
TANES POOMTONG,⁴ AND PRASERT SOBHON¹

¹Department of Anatomy, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand; ²Department of Biology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand; ³Department of Medical Science, Faculty of Science, Burapha University, Chonburi 20130, Thailand; ⁴The Coastal Aquaculture Development Center, Department of Fisheries, Klongwan, Prachuabkirikun Province 77000, Thailand

ABSTRACT The hypobranchial gland of *Haliotis asinina* exhibits major and minor folds that are called leaves and leaflets. When viewed by scanning electron microscope, numerous tufts of rod-shaped cilia, paddle-like cilia, and clusters of granules being exocytosed from the pores were observed on the surface of the leaves and the leaflets. In transverse sections, each of the leaf and the leaflet can be divided into two areas: the basal area consists mainly of two types of large goblet cells, and the apical area that contains a mixture of supporting cells, sensory cells and four types of mucus-secreting cells. *H. asinina* has bipectinate gills. Gills are observed in abalone at the age of 1 mo; and the number and length of their filaments increase with age. In mature abalone, there are about 17 filaments per each gill with equal numbers on both sides. The length of the longest filament is approximately 2.48 mm. Each filament is supported axially by a thin collagenous connective tissue. On the efferent side, there is a V-shaped chitinous skeleton rod. Epithelium lining the filament is composed of tall columnar cells bearing microvilli mixed with ciliated columnar cells and mucus cells.

KEY WORDS: *H. asinina*, gill, hypobranchial gland, histology, SEM

INTRODUCTION

The hypobranchial gland is a single or paired, highly glandular area of the epidermal lining the roof of the mantle cavity (Hyman 1967). Two such glandular areas, one on each side of the rectum, occur in *Haliotis*, and the left one is larger than the right one (Crofts 1929). The hypobranchial gland consists of regular folds or lamellae oriented at right angles to the mantle wall (Hyman 1967). The histology of the hypobranchial gland has been investigated (Crofts 1929, Bevelander 1988). Crofts (1929) described three types of cells in the hypobranchial gland of *Haliotis tuberculata*: mucus cells with spindle shaped secretion, mucus cells with granules, and ciliated cells. Bevelander (1988) described 3 types of cells in the hypobranchial gland of *H. rufescens*: mucus cells with rod-like elements, mucus cells with granular cytoplasm, and supporting cells.

Gills are the principal organs for respiratory gas exchange in mollusks. They are positioned in the mantle cavity (Crofts 1929, Eertman 1996), so they are affected by many substances that flow through the mantle cavity (Schulte-Oehlmann et al. 2000). The studies on gastropod gill morphology is very limited; a few papers have been published on the structure of gills of pulmonate *Siphonaria capensis* (De Villiers & Hodgson 1987) and of some caenogastropod species. The gill structure of the investigated gastropods shows basic uniformity because the gill filaments are composed of a ridge and an extended sheet of nonciliated cells. However, the gill filaments of these various species of gastropods differ in the shape of the filaments (corrugated, triangular, or rounded). Each gill filament is covered with a single layered epithelium of either cuboidal (Schulte-Oehlmann et al. 2000) or columnar cells (Crofts 1929). However, there seems to be a difference in the thickness of the epithelial cells. A hemocoelic space occupies the center of each filament (Eertman 1996, De Villiers & Hodgson 1987). Crofts (1929) found that in *H. tuberculata*, the

V-shaped chitinous skeletal rod, attached to one side of the gill epithelium was similar to that in cephalopoda (Haszprunar 1987). The epithelium of the gill of *S. capensis* and *H. tuberculata* consists of three types of cell: nonciliated cell, ciliated cell, and secretory cell (De Villiers & Hodgson 1987).

To the best of our knowledge, there is still no information on the histology of hypobranchial glands and gills in *H. asinina*, a common abalone species found along the coastal water of Thailand, which is considered to be one of the economic aquatic animals that has been cultured for commercial exploitation. Hence, this study reports on the histology of hypobranchial glands and gills of this species.

MATERIALS AND METHODS

Collection of Abalone Specimens

Abalones were obtained from a land-based culture system at Coastal Aquaculture Development Center, Department of Fisheries, Prachuabkirikun Province, Thailand. They were reared in concrete tanks housed in the shade, and well flushed with mechanically circulated sand-filtered seawater, and provided with an air delivery system to maintain the stable controlled environment. The optimum level of salinity is ~22.5–32.5 ppt, and the temperature ~22 °C to 26 °C (Singhagrainwan & Doi 1993). They were fed with macroalgae (usually *Gracilaria* spp. and *Laminaria* spp.), supplemented with artificial food.

They were anesthetized in 5% MgCl₂, after which their shells were removed. The gills and hypobranchial glands were dissected out and processed for light microscope (LM) and scanning electron microscope (SEM) studies.

Specimen Preparation for LM

Specimens were fixed in Bouin fluid in 0.14 M NaCl for 24 h and washed with 70% ethyl alcohol. Then, they were dehydrated through a graded series of ethanol, cleared in dioxane, infiltrated

*Corresponding author. E-mail: scewn@mahidol.ac.th

and embedded in paraffin. Five-micron-thick sections were cut and stained with hematoxylin and eosin.

For semithin sections, specimens were fixed in Karnovsky fixative (2% paraformaldehyde and 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.8) at 4 °C for overnight, and washed with 0.1 M sodium cacodylate buffer. They were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer at 4 °C for 1 h. Then, they were dehydrated in graded series of ethanol and embedded in Araldite 502 resin. Sections were cut at 1- μ m thickness with Porter Blum MT-2 μ Ltramicrotome, and stained with methylene blue or PAS-methylene blue (Hayat 1993). Examination of the tissues sections was done under an Olympus Vanox light microscope.

Specimen Preparation for SEM

Hypobranchial glands were cut and fixed in a Karnovsky fixative (4% glutaraldehyde 2% paraformaldehyde in 0.1 M sodium cacodylate buffer), pH 7.8 at 4 °C, for overnight, and washed in 0.1 M sodium cacodylate buffer. They were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4 °C. Then, they were dehydrated in graded series of ethanol, and dried in a Hitachi HCP-2 critical point drying machine, using liquid CO₂ as a transitional medium. They were then mounted on aluminum stubs and coated with platinum and palladium in an ion sputtering apparatus, Hitachi E 5000. The specimens were examined in a Hitachi S-2500 scanning electron microscope with an accelerating voltage of 15 kV.

RESULTS

Hypobranchial Gland

The hypobranchial gland of *H. asinina* is located on the dorsal surface of the mantle cavity and runs parallel to the gill. It appears as a large yellow pectinated ridge whose lateral sides are connected to the gills by a thin mantle membrane (Figs. 1A and B, 2A). Each gland consists of many axes, from each of which 8 to 11 leaves arise (Fig. 1B). Under SEM, the major folds or leaves decline from the axis to the lower level and branch into two to three terminals. Many minor folds or leaflets branch off from both sides of each leaf (Fig. 1C). The hypobranchial gland exhibits many tufts of rod-shaped cilia, paddle-like cilia, and granules that are exocytosed from pores on the surface of leaves and leaflets (Figs. 1E and F). In contrast, the junction between the axis and leaf has fewer ciliary tufts, round granules that are being exocytosed from pores on the fold, and there are deep grooves between the folds (Fig. 1D).

In transverse section, each leaf and leaflet can be divided into 2 areas: the basal area, which consists mainly of large mucus cells and the apical area that contains a mixture of supporting cells, sensory cells, and small mucus-secreting cells having goblet appearance (Figs. 2B and C, 3A). These cells have the following characteristics.

1. Supporting cells. These cells vary in shape; they can be triangular, oval, or spindle (Figs. 3B and C). The nucleus is oval and contains mostly pale-stained euchromatin. The upper part of this cell type is wide and shows many long microvilli.
2. Sensory cells. These cells are elongated in shape, and they contain euchromatic oval nuclei with many nucleoli (Figs. 3B and C). The upper part of this cell type is narrow and

reaches the surface of the epithelium where it bears some cilia.

3. Mucus cells. These cells are filled with many mucin granules and have oval shape. Based on the staining characteristics with PAS-methylene blue on semithin sections, there are 4 types of mucus cells (Figs. 3B and C). Type-1 is a mucus cell with a round nucleus and PAS and methylene blue negative granules in the cytoplasm. It is located near the basement membrane (Figs. 3B and C). Type-2 is a mucus cell with flattened euchromatic nucleus and numerous blue granules packed tightly together in the cytoplasm (Figs. 3B and C). Type-3 is a small mucus cell whose cytoplasm is filled with PAS-positive round granules (Figs. 3B and C). Type-4 is the largest mucus cell containing large heterogeneous granules with either pinkish or bluish (methylene blue positive) hues (Fig. 3C).

In the basal area, there are 2 types of mucin cells lying in alternation. Type-1 is the mucus cell, which contain homogenous purple (methylene blue positive) material in the cytoplasm (Fig. 3D). Type-2 is the mucus cell containing homogeneous pale pink (PAS positive) material with streaks of deep pink rod-like materials embedded in the former (Fig. 3D).

Gills

H. asinina has two bipectinate gills, positioned slightly to the left of the center in the mantle cavity and pointing anteriorly (Figs. 1A and B). The gills are attached to the mantle by a thin membrane. There are equal numbers of filaments on both sides (i.e., about 17 filaments per each gill). All filaments lie parallel to each other. Each is a delicate pleat with blunt free tip and is corrugated in the middle (Fig. 4A).

Transverse sections through the gill filaments reveal that they are covered with a single layered epithelium (Figs. 4B to E). Each filament is supported axially by a thin collagenous connective tissue, enclosing the hemocoelic space, which contains hemocytes (Fig. 4E). On the efferent side, there is a V-shaped opaque chitinous skeletal rod (Fig. 4B), serving as the attachment site for muscles that could bring about considerable movement of the gill filament. The gill filament is lined by a columnar epithelium that varies much in thickness in different parts (Figs. 4B to E). Most of the epithelial cells on the proximal and distal ends of a filament are tall columnar bearing microvilli, which appears as brush border under LM, with ciliated epithelial cells (Figs. 4B, C, and E). There are numerous cilia on the region of the apical ciliary band (Fig. 4B) and the lateral ciliary band (Fig. 4C). However, in most of the filaments, the cells are cuboidal and have no cilia (Fig. 4D). Four types of cells can be identified in the epithelium covering the efferent side of the filament (Fig. 4B): (1) the cuboidal cells with round nuclei on the lateral side of the skeletal rod; (2) the ciliated tall columnar cells in the distal end of the filament; (3) the small columnar cells with dense granules and microvilli; (4) the mucus cells with large basophilic or small metachromatic granules in the efferent filament.

The afferent epithelium of the gill filament comprises of 4 types of cells (Fig. 4E). These are: (1) the cuboidal cells with round nuclei with euchromatin and distinct nucleoli on the lateral side; (2) the tall columnar cells with round or oval nuclei located in the terminal epithelium; (3) the ciliated columnar cells in the distal end of the filament; (4) the mucus cell with numerous dense granules packed tightly together in the apical cytoplasm (Fig. 4E).



Figure 1. A & B. Photographs of the dorsal view of the mantle cavity showing the external features of adult *H. asinina* (16 mo old), after removing the shell and dissecting away mantle membrane. ad-adductor muscle, ax-axis, dig-digestive gland, ey-eye, gi-gill, go-gonad, hg-hypobranchial gland, le-leaf, pe-pedal muscle, rt-rectum, te-cephalic tentacle. C. A low-power SEM micrograph of a hypobranchial gland of *H. asinina* showing the leaf (le) which arises from the axis (ax), and leallets (lf) which branch off from both sides of the leaf. D. Higher magnification of a part on the axis from box 1 in C showing ciliary tuft (ct), secreted granules (gr) and exocytosed pores (p) on the tegumental fold. Notice many deep groove (g) between the tegumental fold. E. A high-power micrograph of a part of the leallet from box 2 in C, showing numerous ciliary tufts (ct) that are enlarged in the inset, secreted granules (gr) and exocytosed pore (p). F. A nearby area from box 3 in C at the same magnification, showing many paddle-like (pc) cilia that are magnified in the inset.

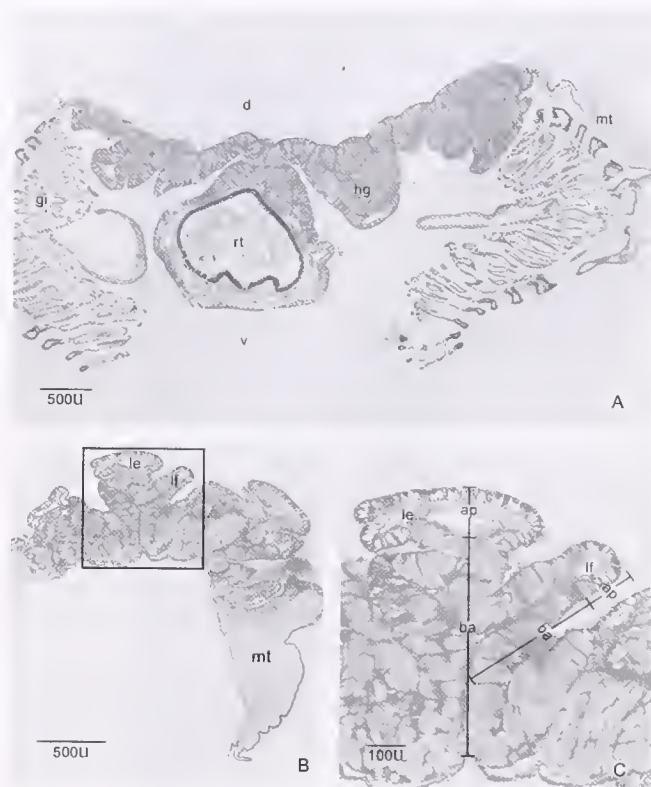


Figure 2. Light micrographs of cross section of the hypobranchial gland. A. A survey photomicrograph showing the location of gill (gi) and hypobranchial gland (hg), which are connected by the mantle (mt). d-dorsal, rt-rectum, v-ventral. B and C. Micrographs showing many leaves (le) and leaflets (lf), whose basal (ba) and apical (ap) parts appear to contain mucus cells of distinct characteristics.

DISCUSSION

Hypobranchial Gland

There are some controversies on the types of cells found in the hypobranchial gland. Crofts (1929) and Bevelander (1988) reported 3 cell types in the hypobranchial gland of *Haliotis tuberculata* and *H. rufescens*, respectively. Both authors described 2 types of mucus cells that contain different secretion. Type-1 mucus cell contains rodlike or spindle-shaped secretion while type-2 mucus cell contains granular secretion (Crofts 1929, Bevelander 1988). The third cell type is the supporting cell (Bevelander 1988), which is ciliated (Crofts 1929). These two types of mucus cells correspond to those found in the basal area of hypobranchial gland of *H. asinina*. The first type of mucus cell contains large, rodlike mucin granules that gave an intense positive reaction to PAS, indicating the presence of neutral mucopolysaccharide. In *H. rufescens*, the mucus cell also contains rodlike granules that were identified to be acid mucopolysaccharide in nature (Bevelander 1988). The second type of mucus cell in the basal area of *H. asinina* hypobranchial gland appears homogenous and stains purple with PAS-methylene blue. This may be equivalent to the second type of mucus cell of *H. rufescens*, but the content appears more homogenous in *H. asinina*. Bevelander (1988) suggested that the granules in these cells were glycoprotein.

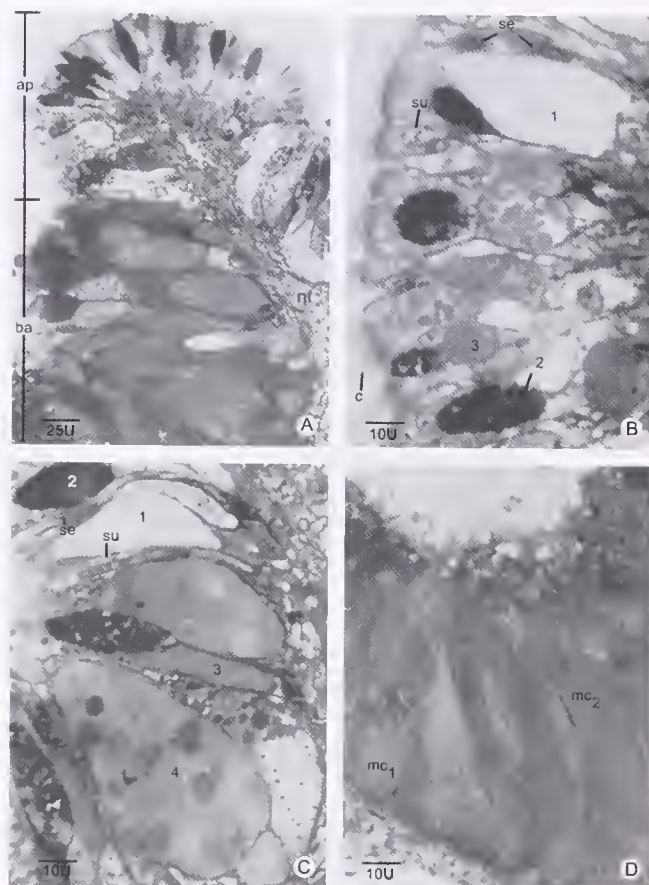


Figure 3. Semithin cross sections of the leaf of hypobranchial gland stained with PAS-methylene blue. A. A micrograph showing apical part (ap) and basal part (ba) which contain different types of mucus cells and nerve fiber (nf) in the core of the leaflet. B and C. High-power micrographs of the apical part showing supporting cell (su), sensory cell (se) and 4 types of mucus cells: type-1 (1), type-2 (2), type-3 (3), type-4 (4). D. A high-power micrograph of the basal part, showing two cell types of mucus cells lying in alternation; the first cell-type is PAS positive mucus cell (mc_1), and the second cell type (mc_2) methylene blue positive cell.

In *H. asinina*, the apical areas of hypobranchial gland leaves and leaflets represent specialized zones where at least 6 types of cells are formed, (i.e., supporting cell, ciliated sensory cell, and 4 types of mucus cells) whose classification is based on the appearance and staining characteristics of the granules. These mucus cells appear very different from those found in the basal area, both in cell shape and secretion. Judging from the staining pattern to PAS-methylene blue, type-1 and type-3 mucus cells contain neutral mucopolysaccharide granules, whereas type-2 cells contain basophilic granules suggesting acidic protein in mature (Humason 1972). Type-4 mucus cell contains both types of granules, neutral mucopolysaccharide and basophilic granules.

Because of the vast number and considerable variety of mucus cells, the principal function of hypobranchial gland should be the secretion of mucin (Hyman 1967). The surface area of the gland is increased by the folding of the glandular epithelium into large pleats to increase the mucus-secreting area. The quantity of mucus

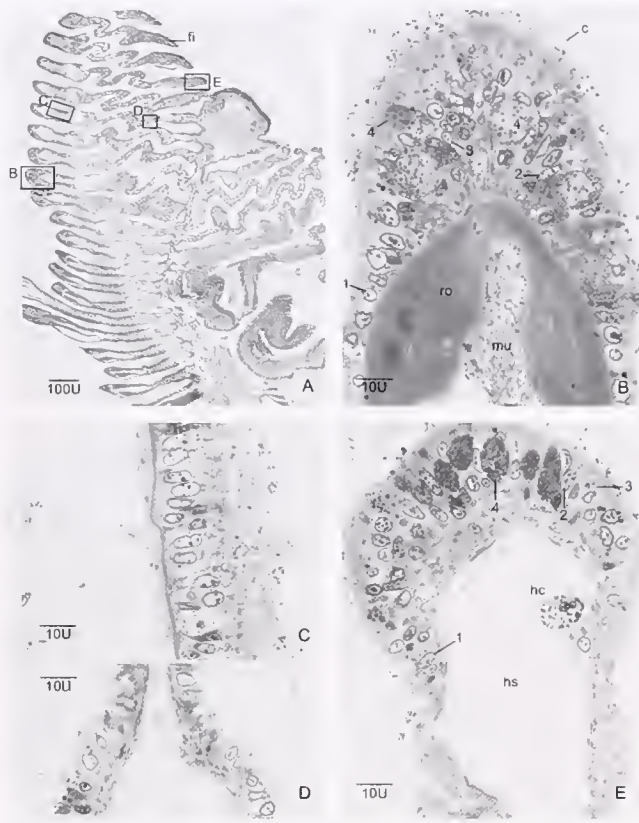


Figure 4. Photomicrographs of the gill of *H. asinina*. A. A micrograph of the gill showing many filaments (fi) aligning parallel to each other. B. An enlargement from the efferent end of the filament from box B in Fig. A, showing four types of cells (1–4) in the terminal epithelium which possess both microvilli and short cilia. C. A bundle of muscle (mu) is attached to the V-shaped skeletal rods (ro). C and D. High-power micrographs of the epithelium of the gill taken from boxes C and D in Fig. A, showing many high columnar cells with long cilia in the epithelium near the efferent side and low columnar or cuboidal cells in the epithelium at the middle of the filament. E. An enlargement from the afferent end of the filament taken from box E in Fig. A, showing four types of cells in the epithelium (1–4). A hemocyte (hc) is seen in the hemocoelomic space (hs).

discharging into the respiratory chamber increases suddenly if the animal is irritated. Mucus is thus produced and secreted for the protection and for clearing away debris from the anus and renal organs, to keep the gills and mantle cavity clean. Furthermore, when foreign irritating particles from turbid water are attached to the surface of the gland, the mucus cells may release mucus to bind particles that will be brushed away by the ciliary action of the epithelial cells. The mucus cells that perform this function may be type-2 mucus cells because they have similar characteristics to those observed in the gill epithelium that may perform similar function. Crofts (1929) found that irritating oils introduced into the entrance of the respiratory chamber seemed to be perceived at once by the hypobranchial gland, and the shell closed down abruptly, and at once a large amount of mucus was released from the mucus cells. In a similar experiment, Alexander (1970) introduced milk into the respiratory chamber, and obtained similar responses. Furthermore, some mucus cells may release mucin into the seawater, perhaps to clear away the offending substance, as well as to adjust pH of seawater to be suitable for respiration. The mucus cells

participating in this protective action may belong to 2 types (i.e., the acidic and basic mucus cells types-1 and 3). The remaining mucus cell (type-4) may act as general mucus cells that produce mucin to lubricate the organ and protect the gills. Furthermore, the heterogeneity of the mucus cells of hypobranchial gland implies that the secretion of mucin for clearing irritants and debris may not be the only function. The complexity of the mucin released could be involved in other process, such as acting as the inducers for the larval settlement, because it has been shown that mucus trail from adult animals is one of the most important factors for settlement.

Gill

Light microscopic observation shows that the internal structure of the gills and the gill filaments of *H. asinina* have an internal architecture similar to those of other mollusks. The gills are bi-pectinate, with individual filaments showing basic similarities. All filaments are positioned parallel to each other and are linked by a common base through which the hemolymph is directed and distributed to the individual filaments (Eertman 1996). Each filament is corrugated in the middle part, which encloses the hemocoelomic space, thus it may be a modification to enlarge the total gill surface area and improve respiratory gas exchange (Crofts 1929, Eertman 1996).

The epithelium of the gills in *H. asinina* is a simple columnar or cuboidal type, which presumably helps to enhance the rapid gas exchange. This feature has been found in most species of mollusks studied so far (Crofts 1929, Eertman 1996, De Villiers & Hodgson 1987). The gill filaments possess areas of ciliated cells alternating with areas of nonciliated cells. In our study, the efferent margin of the filament and both sides of the hemocoelomic space in the efferent side consist of ciliated cells (Crofts 1929). Ciliary movement may take part in sweeping mucus secretions from the mucus cells that serve to capture foreign particles and remove them from the gills (Nuwayhid et al. 1978). Hence, the ciliated cells lining the gills observed in the present study may play both roles in making water current and removing irritating particles.

Nuwayhid et al. (1978) did not find any mucus cells in the gills of *Patella vulgata*, whereas in *Siphonaria capensis* there were a larger number of these cells (De Villiers & Hodgson 1987), and in *Anstrocochlea constricta* there were two types of mucus goblet cells (Eertman 1996). The present study revealed two types of mucus cells on the gills of *H. asinina*. All of them are grouped at the efferent and afferent sides of the filament. It was suggested that these mucus cells function primarily in the cleaning of gills by removing dirt in coordination with muscle contraction and ciliary movement (Yonge 1952). The chitinous skeletal rods found in the efferent side may serve for attachment of muscles that bring about considerable movement of the gill filament (Crofts 1929, Haszprunar 1987). In *S. capensis*, it was found that the muscle fibers were located at intervals on the hemocoelomic surface (De Villiers & Hodgson 1987). On the contrary, the present study reveals that the muscle fibers are attached to the inner surface of the rod. The function of this muscle may be the same as described earlier.

ACKNOWLEDGMENTS

This research was supported financially by the Thailand Research Fund to Prasert Sobhon for a Senior Research Scholar Fellowship.

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BIOLOGICAL ZERO POINT IN HYBRID PACIFIC ABALONE

HONGEN ZHAO, JINSHI ZHANG, LIHONG HUANG, AND LIMIN SUN

Dalian Aquatic Products Research Institute, Dalian, 116000 China

ABSTRACT The biologic zero point (BZP) of hybrid Pacific abalone *Haliotis discus hannai* (China) and *Haliotis discus discus* (Japan) at temperatures of 16°C, 20°C, and 22°C was calculated from the developmental hatching rate, formation of larval retractor muscle, 90 degree torsion and formation of epipodial tentacles. BZP for the early development of hybrid Pacific abalone is 6.22°C, which is an important result for the hybrid breeding of Pacific abalone.

KEY WORDS: hybrid, Pacific abalone, biological zero point

INTRODUCTION

Pacific abalone, *Haliotis discus hannai* is a key aquaculture organism, cultivated in China since the 1980s. Commercial seed production commenced in 1987 and, from this time to 1992, abalone aquaculture grew greatly. Most research was focused on development of hatchery seed production techniques and grow-out modes. A series of key techniques involving spawning, larval rearing, and juvenile and seed nursing were established, as well as grow-out systems. Disease and abnormal mortality has recently, however, severely disrupted the industry and promoted the hybridization of *Haliotis discus hannai* (China) and *Haliotis discus discus* (Japan). Hybrid F₁ shows a significant heterosis in survival and growth and is playing an important role in Chinese abalone culture.

A successful culture and high yield are assured by altering rearing conditions at three important stages during larval development: hatching, development of larval shell, and settlement (Hahn 1989). Information on BZP and effective accumulative temperature (EAT) is necessary for the culture of broodstock and hatchery management. BZP is one of the values used to calculate EAT. BZP varies among abalone species, depending on water temperature in each location. The BZP has so far been investigated in seven abalone (Sawateerap et al. 2001) and this study investigates the BZP of hybrid F₁ of Pacific abalone.

MATERIALS AND METHODS

Conditioning of Broodstocks

Mature broodstock of *Haliotis discus hannai* and *Haliotis discus discus*, with a shell length of 8 cm and a weight of 140–180 g, were used. Males and females were separately reared in different nets and placed in 0.7 m³ plastic tanks. Filtered seawater was used and changed daily. Rearing temperature was maintained at 20°C with constant aeration. Broodstock were fed with fresh kelp.

The dietary grazing rate was calculated daily and gonad maturation was determined by size and color according to Ebert and Houk. (1984).

Induced Spawning

Spawning was induced in April 2000. Individuals with mature gonads were chosen for spawning. Female abalone was dried in a shade room (24°C) for 1.5 h, males for 40–60 min. Broodstock were separated, one by one, into 20-L tanks with filtered water irradiated by ultra-violet at 700 mw.L/h. At the same time, abalone for spawning was induced with flow-through seawater at 1–2 L/min and from 24°C to 22°C. Spawning occurred after about an hour.

Artificial Fertilization

Eggs and sperms were collected separately. Eggs were placed in the experimental container at a density of 6 eggs/mL and then maintained at different temperatures (16°C, 20°C, 22°C). Each experiment was performed in triplicate.

Rearing Management

The zygote was washed once every hour with sand-filtered seawater. After hatching, healthy larvae were selected for continued rearing and water was changed every 8 h.

Observation

Larval development was observed microscopically every hour until the epipodial tentacle stage. Development rate of hatch-out, formation of larval retractor muscle, 90 degree torsion and formation of epipodial tentacle were recorded.

Data Analysis

The relationship of rate (t) of larval development and water temperature was $1/t = AT + B$ provides the basis for BZP calculation.

RESULTS

Experiments were repeated three times and data is showed in Table 1.

The BZP was calculated at water temperatures of 16°C, 20°C and 22°C. The average BZP was 6.22°C (Table 2).

TABLE 1.
Developmental rate of hybrid larvae of Pacific abalone at water temperature 16, 20, 22°C.

Group	C	Time of Larval Development (h)			
		Hatch-Out	Larval Reactor Muscle	Torsion (90°)	First Epipodial Tentacle
1	16	17.6	30.9	44.4	87.1
	20	11.8	19.4	31.5	62.5
	22	10.4	19.3	27.4	54.8
2	16	17.7	30.8	44.4	87.1
	20	11.8	20.2	31.5	65.5
	22	10.7	19.8	27.4	54.8
3	16	17.8	31.4	44.4	87.1
	20	12.5	20.0	31.5	64.5
	22	11.5	19.2	27.4	54.8

TABLE 2.

Relationship between water temperature and time on the formation of the fourth larval developmental stage (1/time) and BZP of hybrid abalone.

Larval Development Stage	Equation of Relationship	BZP
Hatch out	$1/t = 0.00604T - 0.0395$	6.54
Larval retractor muscle	$1/t = 0.00340T - 0.0211$	6.21
Torsion (90-degree)	$1/t = 0.00232T - 0.0146$	6.29
Epipodial tentacles	$1/t = 0.00113T - 0.0066$	5.84
Average		6.22

t = hours, T = °C

DISCUSSION

BZP provides the day/hour when larvae are best ready for washing, selection, and settling and is necessary, therefore, for the effective handling of fertilized eggs and larvae in abalone seed production. It also provides a means of obtaining synchronously developed material for this species and presumably others, for developmental, physiologic, ecologic and biochemical investigations.

BZP varies among species, depending on specific geographical location (Table 3). Moreover, the same species in different geographical locations, also has different BZP, hence *H. discus hannai* of Japan is 7.6°C and *H. discus hannai* of China is 4.2°C (Zhao 1999).

TABLE 3.

The biological zero degree of some abalones.

Species	Biological Minimum Zero (°C)	References
<i>H. discus hannai</i>	7.6	Seki and Kan-no (1977)
<i>H. gigantea</i>	9.0	Seki and Kan-no (1977)
<i>H. rufescens</i>	8.5	Seki and Kan-no (1977)
<i>H. fulgens</i>	9.9	Leighton (1974)
<i>H. discus</i>	8.5	Hahn (1989c)
<i>H. asinina</i>	15.0	Saowapa (2001)
Hybrid F_1	6.22	The present study

In this experiment, abalone larvae were reared at different temperatures. From the equation of the relationship between water temperature and time for larval development, we know that the larval growth rate shows large discrepancies at different temperatures. Lower water temperature leads to slower development rate and higher mortality. Water temperature is an important factor in many stages of larval development (Seki & Kan-no 1977). In this study, reared larva were reared at a water temperatures of 13°C and 14°C, but the larval mortality was so high that the expected result was not reached. A water temperature of 22°C is optimum for larval development and, therefore, to reduce mortality and improve larval quality, reared larvae should be transferred as soon as possible to high temperature.

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INDUCTION OF GYNOGENETIC DIPLOIDS IN THE SMALL ABALONE, *HALIOTIS DIVERSICOLOR SUPERTEXTA*

MINGYI CAI,^{1,2} CAIHUAN KE,^{1,2,*} ZHIYONG WANG,³ SHIQIANG ZHOU,² ZHAOXIA ZHANG,¹ YILEI WANG,³ AND ZIPING ZHANG⁴

¹Key Laboratory of Marine Environmental Science (Xiamen University), Ministry of Education, Xiamen, People's Republic of China 361005; ²Department of Oceanography, Xiamen University, Xiamen, People's Republic of China 361005; ³Institute of Aquaculture Biotechnology, Jimei University, Xiamen, People's Republic of China; ⁴Department of Biology and Chemistry, City University of Hong Kong, Hong Kong, People's Republic of China

ABSTRACT The conditions for induction of gynogenetic haploids and restoration of gynogenetic diploids, were studied in the small abalone, *Haliotis diversicolor supertexta*. Gynogenetic diploids were produced by first using chromosome inactivated sperm to fertilize normal eggs and then suppressing the second meiotic division of these eggs. Sperm chromosomes were effectively inactivated with UV irradiation for 50–90 sec at an intensity of $1075 \mu\text{W cm}^{-2}\text{s}^{-1}$. The decreased capacity of UV irradiated sperm to fertilize eggs could be improved to an acceptable level by increasing insemination concentration of sperm. Eggs fertilized with genetically inactivated sperm developed into abnormal larvae in the haploid state. The diploids were restored when the gynogenetically activated eggs were treated with 0.5 mg l^{-1} cytochalasin B (CB) for 10 min from 7 min to 17 min post insemination.

KEY WORDS: gynogenesis, abalone, *Haliotis diversicolor supertexta*, UV irradiation

INTRODUCTION

Gynogenesis refers to the development of eggs fertilized by sperm, which genetic material does not contribute to the resulting embryo. Possible applications of the techniques include rapid establishment of inbred lines or strains with high degree of homozygosity, sex-control, and accelerated elimination of recessive deleterious genes from aquaculture population (Arai et al. 1984). Gynogenetic diploids can be obtained by first using chromosome inactivated sperm to fertilize normal eggs and then suppressing the first or second meiotic division of these eggs or the first mitotic division of the zygotes.

In molluscs, studies on gynogenesis were preliminary, and the progress was slow. Arai et al. (1984) first studied on the conditions of inactivating sperm by ultraviolet ray (UV) in *Haliotis discus hannai*. After 6 y, Fujino et al. (1990) induced gynogenetic diploids in *Haliotis discus hannai* by cold shock treatment of zygotes after insemination with UV-irradiated sperm, which resulted in 50% to 60% gynogenetic diploid in 6-mo-old individuals proved by the analyses of isozyme genotypes. In the pearl oyster *Pinctada martensii*, Xu et al. (1990) produced high levels of gynogenetic embryos, but did not report the production of viable postlarval gynogens. In the Pacific oyster *Crassostrea gigas*, Guo et al. (1993) produced gynogenetic diploids using cytochalasin B (CB) treatment of zygotes after insemination with UV-irradiated sperm. Guo and Gaffney (1993) observed the growth of the 10-mo-old surviving individuals of the gynogenetic Pacific oyster. Gynogenetic diploids several weeks old have also been reported in *Mytilus edulis* (Fairbrother 1994) and *Mytilus galloprovincialis* (Scarpa et al. 1994).

Small abalone, *Haliotis diversicolor supertexta*, is one of most important abalone species cultured in China, especially in southern China. Reports concerning the induction of triploids in the small abalone have been published (Yan et al. 1999, Yan & Chen 2002, Yang et al. 1998), whereas studies concerning the technique of induction of gynogenesis in the small abalone have not been re-

ported. Therefore, the purpose of this work is to establish the procedure to induce gynogenetic diploids in the small abalone.

MATERIALS AND METHODS

Gamete Preparation

Samples of sexually matured *H. diversicolor supertexta* (introduced from Taiwan) were obtained from Dadeng Abalone Hatchery near Tongan Gulf, Xiamen, China. Spawning of gametes was artificially induced by stimulation with air exposure and UV-irradiated seawater. The concentration of the sperm stock solution was determined using a hemocytometer under a microscope. Eggs were rinsed with $0.22\text{-}\mu\text{m}$ membrane filtered sea water, and allotted into $20\text{-}\mu\text{m}$ screens, 2,000–5,000 eggs per screen, ready for fertilization. The following experiments were carried at 22°C to 23°C .

Inactivation of Spermatozoa

To determine optimal UV dosage, sperm was exposed to UV irradiation for various durations. The UV source was two 15-watt germicidal lamps (wave length: 254 nm). Before irradiation, the UV lamps were warmed up for stabilization. Sperm suspension at a concentration of $5 \times 10^6 \text{ sperm mL}^{-1}$ was layered onto the Petri dish on a shaker to form a thin layer of 1-mm depth, and exposed to the UV light for varying duration from 0 sec to 90 sec. Sperm suspension was shaken at a speed of $40 \text{ cycle min}^{-1}$ (THZ-C, Taichang, China) when exposed to UV irradiation. The distance between the lamps and the dish was adjusted to 17.5 cm. At this distance, the intensity of UV was measure as $1075 \mu\text{W cm}^{-2}\text{s}^{-1}$ (UV-B, Handy, China).

After irradiation, 2-mL sperm solution was immediately mixed with eggs in 50 mL sea water. A portion of eggs to which sperm suspension had not been added was used as a blank control to monitor the contamination of normal sperm. At 8 min postinsemination (p.i.), the zygotes were rinsed with membrane filtered sea

Corresponding author. E-mail: chke@xmu.edu.cn

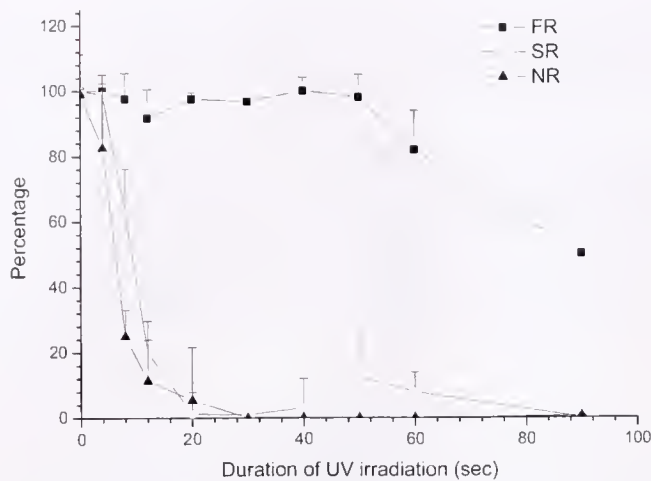


Figure 1. Relationships between UV irradiation duration and the rates of fertilization (FR), survival (SR) and normality (NR) in *H. diversicolor supertexta*.

water and then incubated at 22°C to 23°C. Each group was triplicated in the experiment.

Fertilization rate were calculated by counting the cleaved egg at 1.2 h p.i., and survival rate and normality rate were estimated at 28 h p.i. by examining the morphologic appearance and swimming behavior of larvae. The ploidy of trochophores from each group was determined by chromosome counts in accordance with Li et al. (2000a). The chromosome number of diploid in the small abalone is 32 (Arai et al. 1998).

Optimization of the Insemination Concentration of UV Inactivated Sperm

Results of preliminary experiments indicated that sperm's abilities of motility and fertilizing the eggs were significantly reduced with increasing UV dose, but the fertilization ability could be compensated by improving insemination concentration of sperm. Because 50–90 sec UV irradiation at $1.075 \mu\text{W cm}^{-2}\cdot\text{s}^{-1}$ inactivated sperm effectively, the insemination concentration of sperm

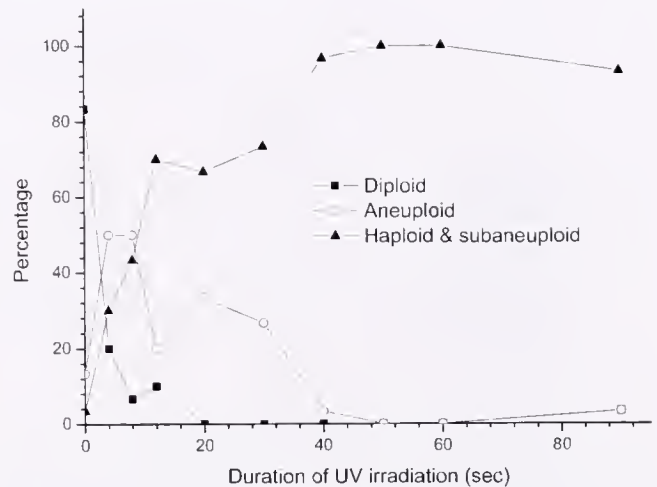


Figure 3. Ploidy of trochophores resulting from fertilization when sperm were exposed to UV irradiation of $1075 \mu\text{W cm}^{-2}\cdot\text{s}^{-1}$ for various duration.

was optimized from 50, 70, 90, and 120 sec irradiation groups in this experiment. The irradiated sperms were mixed with normal eggs with varying insemination sperm concentrations of 5.0×10^4 , 2.5×10^5 , 7.5×10^5 and 1.25×10^6 sperm mL^{-1} . Cleavage of eggs was determined at 1.5 h p.i. Hatching and normality rates in trochophores were determined at 10 h p.i. Chromosome counts were conducted as earlier mentioned.

Production of Gynogenetic Diploids

There were five groups in this experiment. The first group was a blank control group (BC group), in which eggs to which sperm had not been added, were used to monitor the contamination of normal sperms. The second group was the gynogenetic haploid group (G-n group), in which eggs fertilized by inactivated sperms were allowed to develop to haploids without further treatment. The third group was the gynogenetic diploid group (G-2n group), in which gynogenetically activated eggs were treated with CB for chromosome doubling. The fourth group was the normal diploid

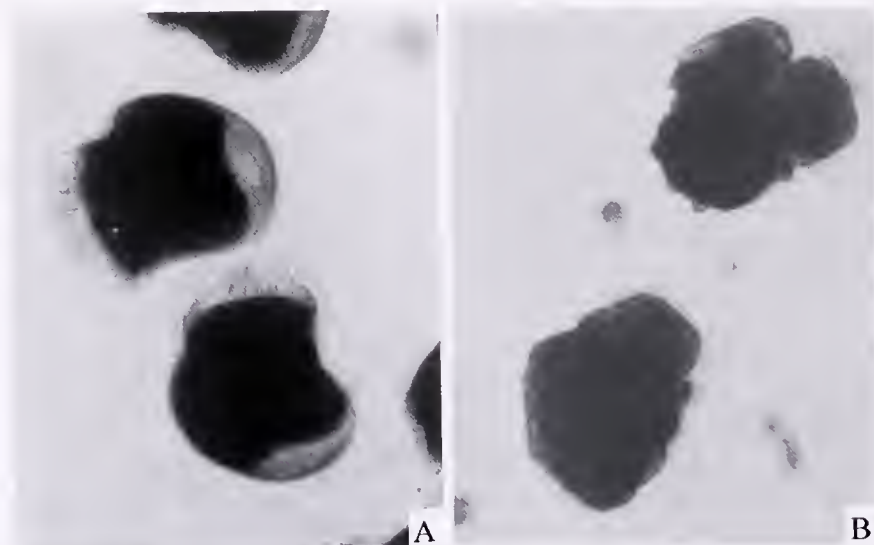


Figure 2. Morphology of normal diploid A, and abnormal haploid B, veligers of *H. diversicolor supertexta* measured at 16 h post insemination.

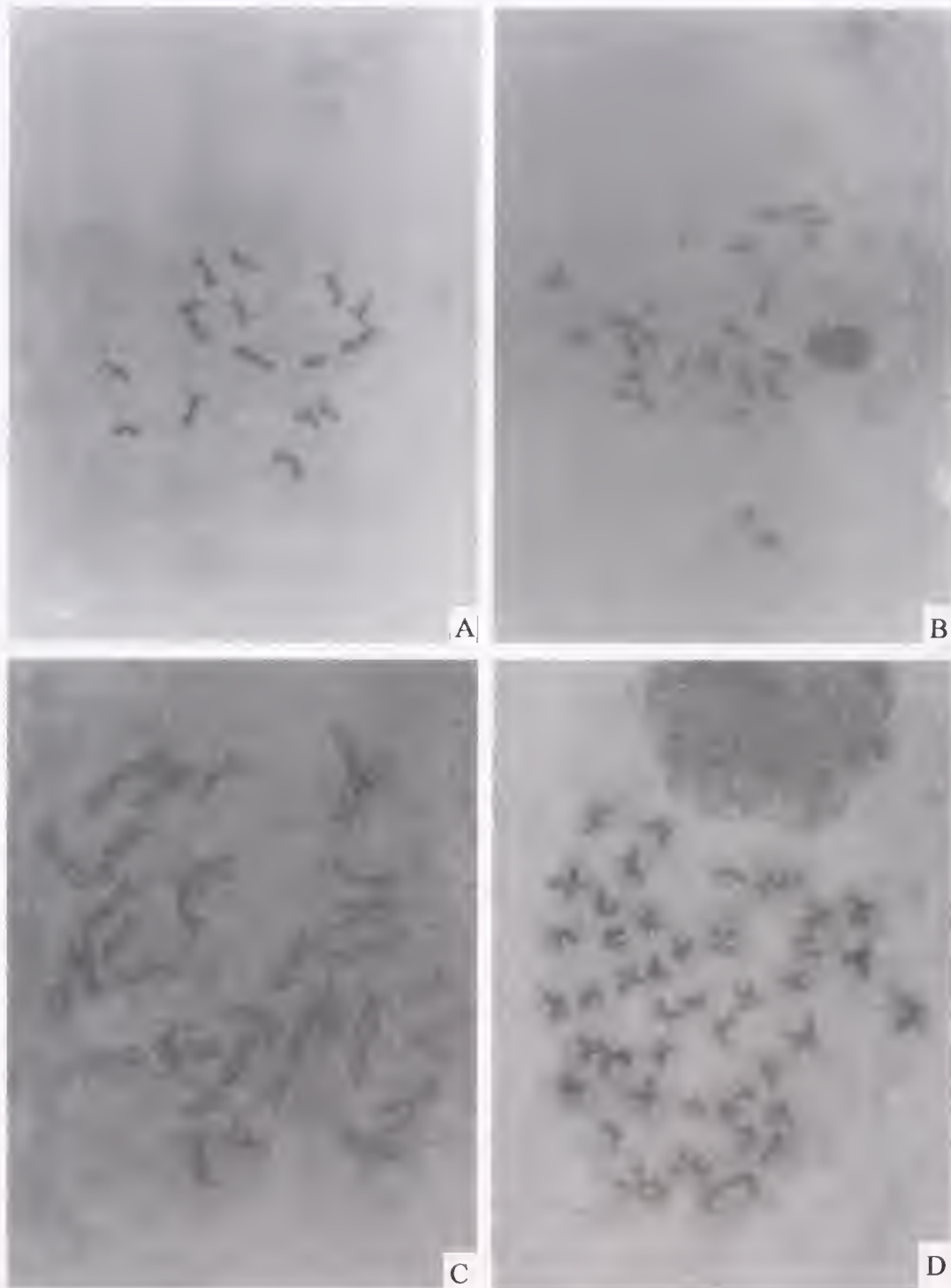


Figure 4. Metaphase plates from haploid A, aneuploid B, diploid C, and triploid D trochophores of *H. diversicolor supertexta*.

group (N-2n group), in which eggs were fertilized with normal sperm without further treatment. The fifth group was normal triploid group (N-3n group), in which eggs were fertilized with normal sperm and treated with CB. This experiment was carried out at 28.6°C. Each treatment was triplicated.

In gynogenetic groups, sperms were exposed to 75 sec UV irradiation based on the results of earlier experiment. Irradiated sperms and normal sperms were used to fertilize eggs with sperm

concentrations of 1.25×10^6 and 5.0×10^4 sperm mL^{-1} respectively. The method for chromosome doubling was conducted according to Yan et al. (1999) with slight modification as follows. The eggs were exposed to 0.5 mg l^{-1} CB (in seawater with 0.033% DMSO) for 10 min. CB treatment started when the first polar body appeared in 50% of the eggs, judged by microscopic examination (7 min p.i. in gynogenetic group, 6 min p.i. in normal group). After CB treatment, the eggs were immersed into seawater with 0.033%

DMSO twice, for 10 min each time, to remove residual CB. Fertilization, hatching, normality rate, and ploidy state were estimated as earlier.

RESULTS

Inactivation of Sperm Chromosomes

The relationship between the duration of UV irradiation and fertilization rate of eggs, survival rate, and normality rate of zygotes at 28 h p.i. is shown in Figure 1. The fertilization rate was 100% for the 0 sec group, and did not decline apparently less than 60 sec irradiation, whereas the rates of survival and normal development of veliger decreased sharply with the increase of UV irradiation. Various kinds of abnormal larvae (Fig. 2b) presented in UV treated groups (e.g., no shell, no velum, small body, disassembled, or agglomerated) whereas the normal larvae (Fig. 2a) that developed into veligers possessed the velum and shell.

The percentage of diploids dropped sharply with increasing duration of irradiation and reached zero by 20 sec irradiation (Fig. 3). A peak of aneuploid formed in short term irradiation groups (4–8 sec) and declined with prolonged irradiation. The percentage of haploid and subaneuploid (chromosome number less than 16) increased steadily and reached 100% by 50 sec UV irradiation. Declining chromosome numbers in the cells of aneuploids were also found. Mitotic metaphase plates from the trochophore cells of *H. diversicolor supertexta* are shown in Figure 4. Dicentric, trivalent, bridged, and fragmental chromosomes occurred in high frequency in the groups of 12–20 sec irradiation.

Insemination Concentration of Inactivated Sperm

The fertilization rates could be improved by increasing the insemination concentration of genetically inactivated sperm (Fig. 5) and reached 100% for 50- and 70-sec groups and 86% for the 90 sec. group, but the sperm from the 120-sec group could not activate eggs even with 12.5×10^5 sperm mL^{-1} . The increase of insemination sperm concentration did not influence the ploidy state of the treatment groups (Table 1). No normal larvae were observed in any treatment groups in this experiment.

Production of Gynogenetic Diploid and Normal Triploid

The fertilization and hatching rate in all treatment groups were high (Table 2). The morphologic normality rate of trochophores was 0% in the G-n group, and increased to 76.6% with CB treatment in the G-2n group.

The distributions of chromosome numbers in trochophore cells from the treatment groups are shown in Figure 6 and the karyological data are presented in Table 3. In the G-n group, diploids were undetectable, 18.8% subaneuploid, 56.3% haploids, and 25.0% aneuploid with low chromosome number (17–21) were observed, which indicated that the UV treatment of sperm, used in this experiment, effectively destroyed the integrity of the sperm genome.

The CB treatment successfully doubled the chromosome number in the G-2n and N-3n groups. In the G-2n group, among the cells observed, 38.6% were diploids, 11.4% were triploids, 4.5% were tetraploids, and 45% were aneuploids with various chromosome numbers (Table 3). No haploid were detected in the G-2n groups. In the N-3n group, 54.8% triploids were produced, 6.5% remained as diploids, and 38.7% were aneuploids.

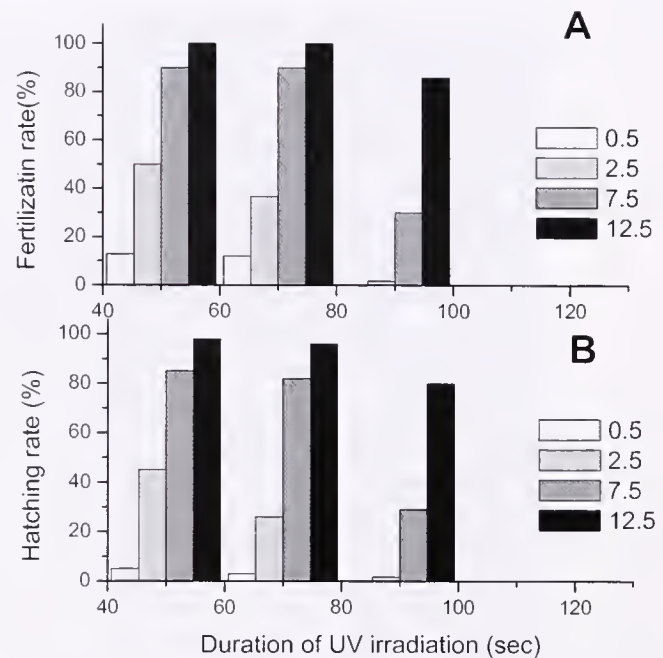


Figure 5. Fertilization rates A, and hatching rates B, of eggs after fertilized with inactivated sperm at various concentration, with various dose of UV irradiation. 0.5, 0.5×10^5 sperm mL^{-1} ; 2.5, 2.5×10^5 sperm mL^{-1} ; 7.5, 7.5×10^5 sperm mL^{-1} ; 12.5, 12.5×10^5 sperm mL^{-1} .

DISCUSSION

Inactivation of Sperm

UV has been used to inactivate sperm chromosomes for gynogenetic induction in many species of molluscs. The dosage of UV irradiation for inactivating the genetic material of sperm varies among species: 30–60 sec of $42 \text{ erg m}^{-2}\text{s}^{-1}$ in *H. discus hamai* (Arai et al. 1984), 120 sec of $4 \mu\text{W cm}^{-2}\text{s}^{-1}$ in *P. martensii* (Xu et al. 1990), 5–6 min of $1,080 \mu\text{W cm}^{-2}\text{s}^{-1}$ in *C. gigas* (Guo et al. 1993), 2 min of $620 \mu\text{W cm}^{-2}\text{s}^{-1}$ in *Mytilus galloprovincialis* (Scarpa et al. 1994), and 50–60 sec of $720 \mu\text{W cm}^{-2}\text{s}^{-1}$ in *Patinopekten yessoensis* (Li et al. 2000b). The optimal dosage of UV irradiation is that which effectively destroys the sperm chromosomes, but does not significantly reduce the ability of sperm to activate eggs. In this study, the results of chromosome counts and fertilization rate determination showed that 50–90 sec UV irradiation of $1075 \mu\text{W cm}^{-2}\text{s}^{-1}$ could effectively inactivate the sperm chromosome but leave the sperm able to activate the eggs of the small abalone.

TABLE 1.

Percentage of haploid and subaneuploid in trochophores increased with UV irradiation dosage ($P < 0.001$) and were not influenced by insemination sperm concentration ($P = 0.44$).

Concentration (cells mL^{-1})	Percentage of Haploid and Subaneuploid Observed			
	50 sec	70 sec	90 sec	120 sec
0.5×10^5	93.2	100.0	100.0	—
2.5×10^5	92.4	100.0	100.0	—
7.5×10^5	90.6	100.0	100.0	—

Data were analysed by Two-way ANOVA using SPSS version 12.

TABLE 2.

Fertilization, hatching and normality rates of eggs/zygotes in the experimental groups.

Group ^a	FR (%)	HR (%)	NR (%)
BC	0.0 ± 0.0	—	—
G-n	97.7 ± 1.5	99.3 ± 0.6	0.0 ± 0.0
G-2n	99.3 ± 0.6	97.8 ± 1.7	76.6 ± 1.5
N-2n	98.6 ± 1.1	99.6 ± 0.6	89.7 ± 0.6
N-3n	99.3 ± 1.2	98.2 ± 1.8	75.0 ± 1.8

Each value represents the mean ± SD ($n = 3$).^a BC, blank control group; G-n, gynogenetic haploid group; G-2n, gynogenetic diploid group; N-2n, normal diploid group; N-3n, normal triploid group.

Combining the results of ploidy analysis and insemination sperm concentration optimization, 75 sec UV irradiation of $1.075 \mu\text{W cm}^{-2}\cdot\text{s}^{-1}$ was used in the production of gynogenetic diploids in this study. Although 75 sec UV irradiation did not destroy all of the sperm chromosomes as aneuploids remained in G-n group, it affected the genomic integrity of all. Aneuploids were also observed in the gynogenetic haploid group in *C. gigas* (Guo et al. 1993, Li et al. 2000c). Aneuploids, and undetectable chromosome fragments in haploids, may be an important reason for low viability of gynogens in fish (Thorgaard 1983, Ihssen et al. 1990). More detailed optimization of the dosage of UV irradiation, based not only on fertilization rate and the proportion of haploid but also on the viability of gynogens in larval and postlarval stages, would inactivate sperm chromosome more completely and possibly make gynogens more viable.

In some reports, fertilization rates decrease with an increasing dose of UV irradiation (e.g., Arai et al. 1984, Li et al. 2000c), whereas in other reports, fertilization rate decreased only after a threshold dose of irradiation (e.g., Guo et al. 1993, Fairbrother 1994). In the present study, fertilization rate decreased significantly only after irradiation for more than 50 sec at the insemination sperm concentration of 2.0×10^5 sperm mL^{-1} , whereas the fertilization rate kept decreasing at a lower insemination sperm concentration (Cai, unpublished data). On the other hand, the decreased fertilization rate could be improved to a higher level by increasing insemination sperm concentration (Fig. 5). This allowed a relative larger dosage of UV irradiation to be used for inactivating sperm chromosomes more completely.

Chromosome Doubling

CB is the most commonly used to induce "chromosome-doubling" for induction of triploids and gynogenetic diploids in molluscs (e.g., Guo et al. 1993, Yan et al. 1999, Li et al. 2000c). CB treatment also doubled the chromosome successfully in this study (Fig. 6). The normality rates were 0% when the sperm were exposed to UV irradiation for more than 30 sec, whereas it increased to 76.6% after CB treatment. A total of 75.1% cells with less than 16 chromosomes were observed in G-n group, whereas 54.5% with more than 32 chromosomes were observed in the G-2n group. Triploids also showed the chromosome doubling.

The starting time of CB treatment is determined by the developmental speed of the early embryo. Allen and Bushek (1992) recommended the time when 50% Pb I appeared in activated eggs as the starting time of CB treatment to block Pb II in the Pacific oyster. The starting time of CB treatment for chromosome doubling in this study was determined by microscopic observation

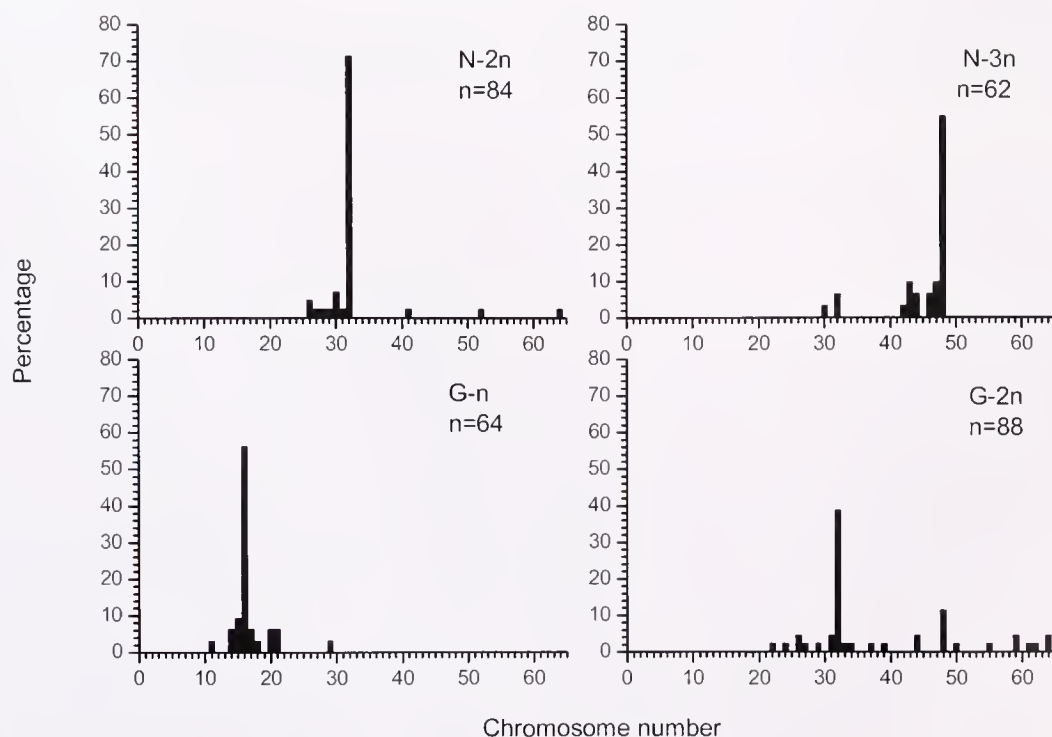


Figure 6. Distributions of chromosome counts in trochophores from various groups. G-n, gynogenetic haploid group; G-2n, gynogenetic diploid group; N-2n, normal diploid group; N-3n, normal triploid group.

TABLE 3.

Levels of euploid (chromosome number) and aneuploid (chromosome number) produced in gynogenetic haploid group (G-n), gynogenetic diploid group (G-2n), normal diploid group (N-2n) and normal triploid group (N-3n)

Groups	Percentage of Ploidies Observed							
	Aneuploid (<16)	Haploid (16)	Aneuploid (17–31)	Diploid (32)	Aneuploid (33–47)	Triploid (48)	Aneuploid (49–63)	Tetraploid (64)
G-N	18.8	56.3	25	0.0	0.0	0.0	0.0	0.0
G-2n	0.0	0.0	18.2	38.6	13.6	11.4	13.6	4.5
N-2n	0.0	0.0	23.1	71.8	2.6	0.0	0.0	2.6
N-3n	0.0	0.0	3.2	6.5	35.5	54.8	0.0	0.0

according to the biologic criteria recommended by Allen and Bushek (1992). The starting time in the G-2n group was later than that in the N-3n group, which was ascribed to the slower developmental progress in comparison with that of normal fertilized eggs (Li et al. 2000a).

Triploids, tetraploids, and aneuploids produced in the gynogenetic diploid group were reported also for the Pacific oyster (Guo et al. 1993, Li et al. 2000c). In this study, the standard deviation of chromosome numbers in G-2n group was much larger than twice of that in G-n group (Fig. 6). The possible reasons for the production of triploids, tetraploids, and large numbers of aneuploids were nonsynchronous development of eggs or inappropriate starting time of CB treatment, which resulted in blocking the release of Pb I, Pb II, or both Pb I and Pb II among eggs. Although chromosome doubling has been successfully induced in this study, further optimization of the starting time, based on ploidy analysis and viability, would improve the yield and quality of gynogens.

The restoration of diploids, determined by karyotyping or flow cytometry, was used to identify the success of genetic induction in fish and molluscs (Guo et al. 1993, Colombo et al. 1995, Li et al. 2000c). In this study, karyotyping was used to monitor the restoration of diploid in genetic induction in the small abalone. Chromosome loss is very common in chromosome preparation, so there

were only 71% diploids in normal diploid group in this study, others being represented as aneuploids after artificial chromosome loss. Because the deviation of the chromosome number from the true one was limited such as in N-2n group, and the majority of distribution of chromosome of chromosome-doubling treatment group could separate from that of no-doubling treatment group (Fig. 6), chromosome counts used in this study still could be used to identify gynogenesis indirectly. The true assessment of the small abalone with exclusively maternal inheritance could only be demonstrated by using biochemical or molecular genetic marker, which was difficult to carry out in larval stage.

Records of viable gynogens in 10-mo-old *C. gigas* (Guo & Gaffney 1993) and in 7-mo-old *H. discus hannai* (Fujino et al. 1990) indicate that it is possible to apply this technique in genetic improvement programs. Studies on the performance of gynogenetic diploids of *H. diversicolor supertexta* are now in progress at our laboratory.

ACKNOWLEDGMENTS

This work was supported by the National High Technology Research and Development Program of China (863 Program, No. 2001AA621080 and No. 2003AA603240).

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POLYETHYLENIMINE PROMOTES SPERM-MEDIATED TRANSGENE AND OLIGONUCLEOTIDE DELIVERY IN ABALONE *HALIOTIS DISCUS HANNAI*

XIAOLONG WANG, JINGJIE HU, JIE PAN, ZHUOJUN MA, KE BI, QUANQI ZHANG, AND ZHENMIN BAO*

Laboratory of Marine Genetics and Breeding, Department of Biotechnology, Ocean University of China, Qingdao 266003, People's Republic of China

ABSTRACT In this study, a pCMV2-EGFP plasmid (Invitrogen), in which the enhanced green fluorescent protein gene under the control of the CMV2 promoter, was used as a reporter gene to evaluate the efficiency of the gene transferring techniques into Pacific abalone *Haliotis discus hannai*. The GFP reporter gene was transferred into abalone eggs with four methods: microinjection method, sperm vector method, polyethylenimine (PEI) method and polyethylenimine-mediated sperm-vector (PEI-SV) method. The results indicate that the PEI-SV method is more efficient and convenient in gene delivery into abalone eggs compared with the microinjection method and the conventional sperm vector method. A significant proportion (13.9%) of the fertilized eggs developed normally into blastomeres and expressed green fluorescent protein at high level after gene transfer with the PEI-SV method, and 7.6% of them survived to the trochophore stage. PCR analysis suggests that the mechanism of PEI-SV gene transfer method was that PEI/DNA was taken up by spermatozoa and carried into eggs during fertilization. In addition, PEI-mediated transport of FAM-labeled 10 mer oligonucleotide into abalone sperm cells resulted in almost 100% bright fluorescent nuclei. The PEI-SV gene transferring method can be an effective tool to transfer gene into other animal species.

KEY WORDS: polyethylenimine, gene transfer, microinjection, sperm, abalone, *Haliotis discus hannai*

INTRODUCTION

Abalone is a valuable marine shellfish with great economic importance in marine aquaculture. *Haliotis discus hannai* is the major commercial abalone species in the northeast coastal area of China. Because abalone grow very slowly, it generally takes 3–4 y of farming from seeding to harvesting, which greatly limits the productivity of abalone aquaculture. Transfer of growth hormone gene is a possible way to breed fast-growing abalone. Research on transgenic abalone is far from successful because of many difficulties in gene transferring technologies. One of the most intractable obstacles is the lack of a suitable method to transfer foreign genes into fertilized abalone eggs.

The first successful transfer of a rat growth hormone gene, under a metallothionein promoter, into a mouse, resulted in a dramatic increase in growth. This was performed using a microinjection method (Palmiter et al. 1982, Palmiter et al. 1983). Since then several other techniques for gene transfer such as sperm vector, electroporation, high-velocity microprojectile bombardment, and sperm electroporation, have been developed. None of these methods are appropriate for transferring foreign genes into marine shellfish species. Because of the high mortality of fertilized eggs and larvae, millions of fertilized eggs must be transferred to ensure the production of transgenic shellfish. Microinjection is still a general method with acceptable efficiency in transferring gene into most animal species. However, it is almost impossible to produce transgenic shellfish with the microinjection method. Generally, micromanipulation cannot produce enough embryos due to the difficulties of skills, the length of time required, and high mortality of the injected eggs. The ease of artificial fertilization of shellfish enables the use of sperm electroporation, a powerful tool for increasing efficiency and number of transgenic embryos. Although high gene transfer efficiency has been reported (Powers et al. 1995), sperm electroporation, as well as microinjection, are difficult to control, demand high skill levels, and are time-consuming methods, which limit their application to shellfish.

To enhance the efficiency and overcome the difficulties in transgenic shellfish research, a new gene transfer method—polyethylenimine-mediated sperm-vector (PEI-SV) is proposed and evaluated by transferring a green fluorescent protein reporter gene into the Pacific abalone, *Haliotis discus hannai*.

MATERIALS AND METHODS

Abalone

Mature male and female parental Pacific abalone *Haliotis discus hannai* individuals were obtained from Rongcheng Xunshan Abalone Hatchery in Shandong Province. After the ovulation or sperm ejaculation of the parental abalone, eggs and sperm were mixed together for fertilization when needed. The density of the eggs and sperm was controlled at approximately 1:10,000 to gain a high fertilization rate. The fertilized eggs were washed with filtered seawater 20 min after fertilization to remove excessive sperm and prevent them from decaying.

The Reporter Gene

A pCMV2-EGFP plasmid (Invitrogen) in which the enhanced green fluorescent protein gene was under the control of the CMV2 promoter, was used as a reporter gene to evaluate the efficiency of the gene transfer techniques. *E. coli* DH5 α was transformed with the pCMV2-EGFP plasmid. The expression of the green fluorescent protein gene in *E. coli* DH5 α was visualized through a fluorescent microscope. The plasmid was then propagated and extracted. Fifty μ g of the circular pCMV2-EGFP plasmid was cut with restriction enzyme EcoR I, purified with a plasmid purification kit (SANGON) and dissolved in 500 μ L of DW₂ for use.

The PEI/DNA Complex

Forty μ g of the linear pCMV2-EGFP plasmid DNA was diluted into 2 ml of DW₂. PEI vector was used at 1:1 PEI/DNA ratios (W/W). The desired amount of 25-kDa polyethylenimine (Sigma) was added to the plasmid solution, mixed, vortexed, spun down, and incubated at room temperature for 10 min before use.

*Corresponding author. E-mail: zmbao@ouc.edu.cn

Gene Transferring

The experiment was designed in 5 groups: control group, microinjection group, SV group, PEI group, and PEI-SV group. The control group consisted of about 50,000 fertilized eggs, without any treatment. The development of fertilized eggs was observed microscopically and expression of GFP was monitored with an Olympus fluorescent microscope. All experiments were done in duplicate. The fertilization and survival rates were calculated for each group at 1 h and 16 h after fertilization, when the fertilized eggs began the first cleavage and became trochophores respectively. The expression of the GFP was monitored with fluorescent microscopy through the developmental process of the embryos from 1 to 6 h after fertilization.

Microinjection Group:

The reporter gene was transferred with the microinjection method. Two μg of the linear pCMV2-EGFP plasmid was diluted with microinjection buffer (7 mM Tris, 0.15 mM EDTA, pH 7.0) to a final concentration of 2 $\mu\text{g}/\text{mL}$. Fertilized eggs were taken from the control group and 500 eggs were injected with 0.02 μL *P_{CMV2-egfp}* solution for each (Fig. 1A). Microinjection was performed using a Nikon micromanipulation system.

SV Group:

About 10,000 eggs were transferred with the conventional sperm-vector method. Four μg of the linear plasmid pCMV2-EGFP was diluted to 2 $\mu\text{g}/\text{mL}$ with DW₂, and mixed with the sperm at 1:50 volume ratio. The sperm/DNA mixture was incubated at room temperature for 10 min and mixed with the eggs for fertilization.

PEI Group:

About 10,000 eggs, taken from the control group, were transferred with the reporter gene using PEI as vector. The DNA/PEI mixture with 4 μg of DNA was mixed with fertilized eggs at a 1:50 volume ratio, 10 min after fertilization.

PEI-SV Group:

About 10,000 eggs were transferred with the PEI-mediated sperm-vector method. The DNA/PEI mixture with 4 μg of the linear plasmid DNA was added to the sperm at 1:50 volume ratio and gently mixed with a pipette. The sperm/DNA/PEI mixture was mixed with the eggs for fertilization immediately, by which the DNA/PEI complex was transferred into the eggs.

PCR Analysis of the GFP Reporter Gene

Polymerase Chain Reaction (PCR) technology was used to testify whether PEI can carry DNA into abalone sperm cells or not. Three sperm/PEI/DNA mixture samples were washed with DW₂ and sperm was collected after centrifuging three times to remove DNA molecules attached to sperm surfaces. Then DNA was extracted with Genomic DNA extraction kit (Sangon) and tested with PCR technology using a pair of GFP gene specific primers: P1: 5'-CCAACACTTGTCCTACTTT-3', P2: 5'-GCTTTGATTCATTCTTT-3'. DNA extracted from normal abalone sperm and sperm/DNA mixture was used as a negative control, and plasmid DNA was used as a positive control. The PCR reactions were performed in a total volume of 20 μL , using 100 nM of each primer, 200 mM dNTPs, in 1X PCR Buffer supplemented with MgCl_2 at a final concentration of 1.5 mM. The amplification was carried out on a MJ PTC-100 thermal controller as follows: a 2 min predenaturing at 94 °C, followed by 30 cycles (30 s at 94 °C, 1 min

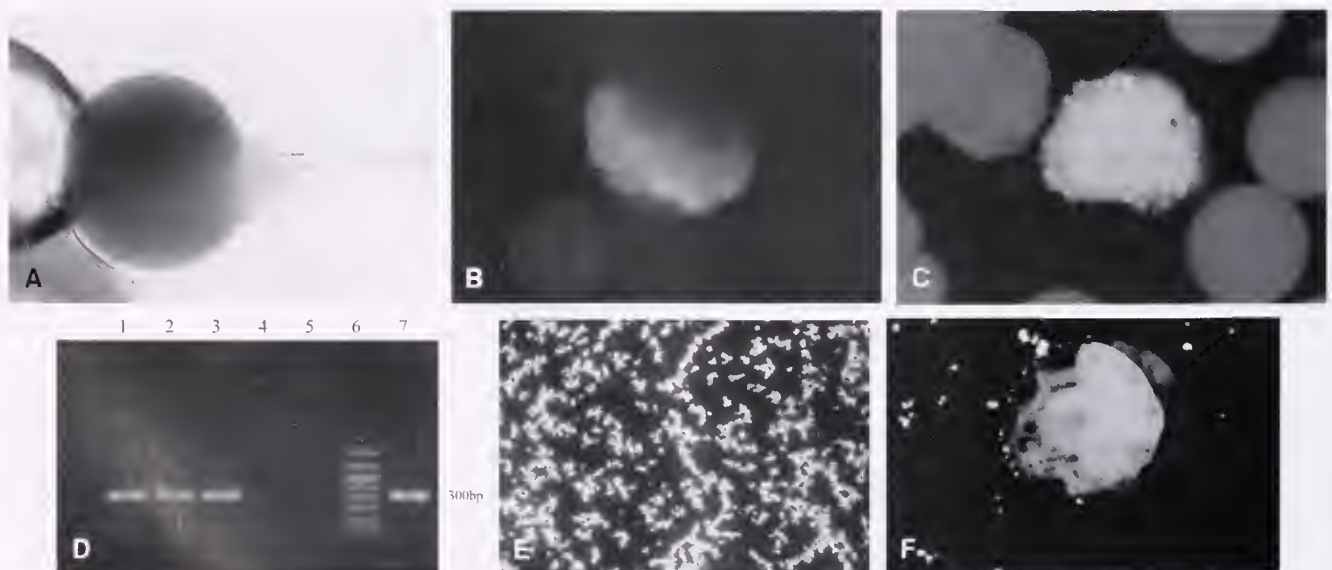


Figure 1. Result of the gene transferring in abalone. A. Gene transferring by microinjection in abalone fertilized egg. B. Mosaic expression of GFP in abalone embryos in PEI group. C. One abalone blastomere in PEI-SV group showing high level GFP expression. D. Agarose gel electrophoresis of PCR products showing that the pCMV2-EGFP plasmid DNA was detected in the sperm cells, lane 1, 2, 3: three samples amplified with sperm DNA extracted from sperm/PEI/DNA mixture washed three times with ddH₂O; lane 4 and lane 5: negative control amplified with sperm DNA extracted from common sperm and sperm/DNA mixture without PEI treatment respectively, Lane 6: 100bp DNA ladder, lane 7: the positive control amplified with pCMV2-EGFP plasmid DNA. E. Sperm cells with green fluorescence transferred with FAM labeled oligonucleotides. F. Sperm cells with green fluorescence attached to the membrane of the fertilized eggs.

TABLE 1.

Fertility rate, survival rate and GFP expression data in each group (%).

Group	Total Number	Fertilization (%)	Survival (%)	GFP Expression (%)
Control	50,000	97.4	83.6	0
Microinjection	500	97.4	0	0
SV	10,000	95.2	82.3	0
PEI	10,000	97.4	52.7	3.8
PEI-SV	10,000	16.3	7.6	13.9

30 s at 60°C, and 3 min at 72°C). The reaction was completed by a final extension step at 72°C for 5 min, then electrophoreses on a 1.5% agarose gel for 30 min at 5V/cm and visualized with UV light after EB staining.

Transferring Fluorescent Labeled Oligonucleotides

Five µg of FAM-labeled 10 bp random sequence oligonucleotides were diluted into 200 µL of DW₂. PEI vector and used at 1:1 PEI/DNA ratios (W/W). The desired amount of 25-kDa polyethylenimine (Sigma) was added to the plasmid solution, mixed, vortexed, spun down, and incubated at room temperature for 10 min. Then the FAM-labeled oligonucleotides and the PEI/oligonucleotides mixture was added to the sperm cells, mixed, vortexed, spun down, and incubated at room temperature for 60 min. The sperm was mixed with eggs for fertilization, and then the sperms and the eggs were observed through fluorescent microscopy.

RESULTS

Fertility, Embryo Survival, and Expression of GFP

The fertilization rate, survival rate and the GFP expression rate for each group is shown in table 1. In the control group, 97.4% of the fertilized eggs cleaved, and 83.6% of them survived to the trochophore stage. The fertilized eggs used in the microinjection group were taken from the control group, so the fertility rate was also 97.4%. However, none of the 500 injected fertilized eggs cleaved 1 h after fertilization. Fluorescent microscopy revealed that none of them expressed GFP at 5 h after fertilization.

In the SV group, 95.2% of the fertilized eggs cleaved and 82.3% of them survived to the trochophore, but none of them expressed GFP. In this study, the conventional sperm vector method did not decrease the fertilization rate and the survival rate, yet it did not deliver GFP gene into the fertilized eggs successfully either. In PEI group, the fertilized eggs were also taken from the control group, so the fertility rate should also be 97.4%, whereas the survival rate to trochophore was only 52.7%, which was significantly lower than that of the control group. Fluorescent microscopy revealed 3.8% of them expressed GFP and a small proportion (less than 5%) of them expressed GFP in mosaic manner (Fig. 1B).

In the PEI-SV group, only 16.3% of the fertilized eggs cleaved by 1 h after fertilization, and 7.6% of them survived to trochophores by 16 h after fertilization. The fertilization rate and survival rate was significantly lower than the control group. The

gene delivery efficiency was, however, significantly higher than the other groups: 13.9% of the fertilized eggs developed into blastomeres and expressed GFP at high level, at 5 h after fertilization (Fig. 1C). Compared with the PEI group and the SV group, it is obvious that PEI promotes the sperm-mediated gene delivery into the abalone eggs.

PCR Analysis of the GFP Reporter Gene

PCR technology was used to test whether PEI could carry DNA into sperm cells or whether it was attached to the cell membrane. The sperm/PEI/DNA mixture was washed three times with clean seawater and the sperm DNA was extracted and amplified with GFP gene specific primers. For all 3 samples tested, the strong PCR bands showed that the PEI/DNA complex did carry DNA into the sperm cell, or the complex attached to the cell membrane that cannot be washed off with water. The negative controls amplified with DNA extracted from normal sperm cells or sperm cell mixed with DNA without PEI treatment, showed no bands, indicating that the sperm cells did not absorb DNA by itself or DNA was degraded by nuclease without the protection of the PEI/DNA complex.

PEI Carried Oligonucleotides to Sperm Cells

Generally short oligonucleotides do not require a carrier to enter a cell through fluid-phase endocytosis. However lysosomal degradation remains a problem. Polyethylenimine has been shown to help oligonucleotides to reach their nuclear target (Boussif et al. 1995). We therefore tried PEI for carrying oligonucleotide into abalone eggs. In the control experiments, abalone sperm cells were incubated with FAM-labeled 10 mer oligonucleotide (10 µM) for 2 h, then rinsed and fixed. Fluorescent microscopy did not reveal any remaining cell-associated oligonucleotide or nucleoside, which may be a consequence of nuclease degradation. In sharp contrast, PEI-mediated transport of the same concentration of FAM-labeled 10 mer oligonucleotide into abalone sperm cells, resulted in almost 100% bright fluorescent nuclei (Fig. 1E). Using these sperm for fertilization, fluorescent microscopy revealed that the sperm with fluorescence attached to the membrane of the fertilized eggs (Fig. 1F), indicating that the sperm are still capable of insemination after PEI/DNA complex treatment.

DISCUSSION

In animals, microinjection was the earliest technique developed to introduce foreign DNA into fertilized eggs. However, it is a difficult and time-consuming technique that requires sophisticated skills. Because of the opaqueness, stickiness, and buoyancy of the embryos, the invisibility of the pronuclei, the toughness of the chorion, and the high mortality of injected eggs, this is not a suitable method for gene transfer in most shellfish species. Gene transfer into abalone fertilized eggs with microinjection technology is extremely difficult because the eggs are surrounded with a tough chorion membrane. If the chorion membrane is removed, the embryos become fragile and are easily destroyed by micromanipulation. The eggs are opaque and the pronucleus is invisible, which makes it almost impossible to inject the DNA solution into the male or female pronucleus of the fertilized eggs, which is generally required in gene transfer with microinjection. The yolk in the eggs easily flows out when the eggs are pierced with the glass micro-

pinhead (Fig. 1A), which could cause the high mortality of the injected eggs. These difficulties also limit the number of eggs that can be injected, even though millions of eggs can be produced by one single female abalone. In this research, the high mortality of the injected eggs and the lack of expression of GFP, indicate that microinjection is not suitable for transferring genes into abalone.

These problems are partially solved by the use of the electroporation method or the sperm electroporation method. High gene transfer efficiency was reported when a recombinant plasmid, containing a beta-galactosidase cassette, was introduced into fertilized eggs of the red abalone *Haliotis rufescens* by electroporation (Powers et al. 1995). The conventional sperm vector method is neither effective nor stable, but it is quite successful when combined with the electroporation method. Sperm electroporation is again a difficult-to-control, and skill-demanding method and its application to marine shellfish is limited because of the high electrical conductivity of seawater. To enhance the efficiency and overcome the difficulties in transgenic shellfish research, here we propose the polyethylenimine-mediated sperm-vector (PEI-SV) method. The results indicate that PEI can promote DNA transfer into abalone eggs, especially when combined with the sperm vector method. A significant proportion (13.9%) of the fertilized eggs developed normally into blastomeres and expressed green fluorescent protein at high level, 7.6% of them survived to the trochophore stage after gene transfer with the PEI-SV method.

PEI is an aqueous organic macromolecule with high cationic charge density potential, which can be complexed with DNA. PEI/DNA complexes can be used for in vitro and in vivo gene delivery approaches. The excess of positive surface charges enhances the association of the complex with the plasma membrane of cells and facilitates their uptake by endocytosis (Wagner et al. 1991). Accordingly, PEI is effective in gene delivery into a variety of cell types even without the addition of cell binding ligands or endosomolytic agents (Wightman et al. 1999, Zatloukal et al. 1999). The in-vivo gene transfer efficiency of these DNA/polycation complexes into tumors also has been assessed (Kircheis et al. 1999). PEI possesses DNA binding and condensing activity, together with a high pH buffering capacity, that is believed to protect DNA from nuclease degradation and to enhance its exit from the endosomal compartment (Bieber et al. 2002, Pollard et al. 1998). Every third

atom of the PEI is a protonable amino nitrogen atom, which makes the polymeric network an effective "proton sponge" at virtually any pH (Boussif et al. 1995). The intracellular transport pathway from the endosome to the nucleus is still not well understood. In this research, the higher gene transfer efficiency of the PEI-SV method, and the detection of reporter gene in the sperm cells, revealed that PEI is also effective in delivering genes into abalone sperm cells. The mechanism is presumably that the PEI/DNA complex is taken up by endocytosis and then carried into the eggs by the sperm cells. Whether DNA is integrated into the nucleus of the sperm cells before or after fertilization is under further investigation.

In the PEI group, the result showed that PEI decreased the survival rate and the gene delivering efficiency was low, which was presumably because the chorion of the abalone fertilized eggs blocked the PEI/DNA complex from entering. The mosaic expressions of GFP suggest that the PEI can deliver genes into one or more cells rather than into all cells of the embryos at two or more cell stages. The fertilization rate and survival rate of the PEI-SV group were significantly lower than the control group, which is presumably because of the lower motility of the sperms caused by the viscosity or cytotoxicity of the PEI. Recently, for its DNA-condensing and pH-buffering properties, PEI has been widely used as a highly efficient nonviral vector in gene therapy for delivering oligonucleotides and plasmids both *in vitro* and *in vivo* (Putnam et al. 2001, Kircheis et al. 1999). However, it was found that high concentrations of PEI/DNA complexes (>6 mg/mL) induced a cellular toxic response in cultured mammalian cells (Boussif et al. 1995). In this study, the lower fertility rate and survival rate of the PEI group and the PEI-SV group, compared with the control group, showed that PEI may be cytotoxic to the abalone sperm cells or embryo cells. Nevertheless, the PEI-mediated sperm-vector method is far more efficient and convenient compared with the conventional methods, and is potentially a powerful tool in transferring genes into other shellfish, fish, and even mammalian species.

ACKNOWLEDGMENT

This work was funded by the national High-tech Youth foundation project (2001AA628050, 2004AA603830) and national key fundamental research and development project (G1999012009).

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IDENTIFICATION OF AN α -PEPTIDE IN *HALIOTIS RUBRA* WITH HOMOLGY TO THE *LYMNAEA* α -CDCP SPAWNING PEPTIDE

SCOTT F. CUMMINS AND PETER J. HANNA*

School of Biological and Chemical Sciences, Deakin University, Geelong, VIC 3217, Australia

ABSTRACT It is now understood that a combination of molluscan reproductive peptides are commonly cleaved from a large preprohormone and influence different aspects of spawning behavior. One type of reproductive peptide, known in *Lymnaea stagnalis* as α -CDCP, and in *Aplysia californica* as α -BCP, acts in egg laying via temperature-dependent autoinhibition or autoexcitation of neuronal cells. In our study, the expression of α -CDCP-like peptide in the blacklip abalone, *Haliotis rubra*, was identified by Western blots and immunocytochemistry, using an antiserum developed against α -CDCP. Western blots of total protein isolated from the central nervous system, cerebral and pleuropedal ganglia, as well as gonad and hepatopancreas tissues of sexually mature adults, identified a protein of approximately 100 kDa as well as a range of smaller reactive peptides. This finding suggests that a reproductive α -peptide is probably synthesized from a single larger precursor protein. The larger peptides were also identified in Western blots of several abalone tissues. Immunocytochemistry using the same antiserum showed the presence of immunoreactive axons in all the tissues studied, indicating synthesis or transport of products. The function of the abalone α -CDCP-like peptide is yet to be determined.

KEY WORDS: *Haliotis rubra*, egg-laying behavior, neuropeptides, α -CDCP

INTRODUCTION

Gastropods have been commonly used to study the neuronal basis of behavior due to the simplicity of their nervous systems. Examples include *Aplysia* and *Lymnaea*, in which reproduction is characterized by a stereotyped behavioral repertoire, controlled by several neuropeptides and culminates in egg laying (Nambu & Scheller 1986, Rothman et al. 1992, Griffond et al. 1992, Van Minnen et al. 1989).

Recombinant DNA technologies have been used to identify and characterize sequences encoding neuropeptides that are associated with the egg laying. These studies have indicated that, like most neuroactive peptides, biologic peptides are initially synthesized and cleaved from a large preprohormone sequence, known as the egg-laying hormone (ELH) preprohormone (Scheller et al. 1982, Scheller et al. 1983, Mahon et al. 1985, Nambu & Scheller 1986). The preprohormone characteristically begins with an initiator methionine residue, a hydrophobic signal sequence, and is followed by a number of cleavage sites (Shyamala et al. 1986). The ELH gene family is defined by the presence of a highly conserved ELH domain, and in *Aplysia* this corresponds to approximately 36 amino acids, while β -, α -, γ -, and calluxin-related bag cell peptides (BCPs) may be present or absent. The *A. californica* gene family consists of five genes, three of which have been well defined, including a BC precursor isolated from BCs and peptides A and B precursors preferentially expressed in the atrial gland (Scheller et al. 1983). Immunolocalization studies have indicated that ELH expression is not restricted to the BCs and the atrial gland because the abdominal ganglion proper, the pleural, cerebral, and buccal ganglia have also been implicated as immunoreactive cell tissues (Shyamala et al. 1986).

It is now understood that, in *Aplysia*, a combination of peptides can be produced from a prohormone and each can influence a different aspect of behavior (Dudek & Tobe 1978, Shyamala et al. 1986, Geraerts et al. 1988). One important peptide derived from the precursor, α -BCP, is produced by the neurosecretory BCs upon

excitation, and acts via a temperature-dependent autoinhibition or autoexcitation of the BCs (Rothman et al. 1983, Sigvardt et al. 1986, Redman & Berry 1991). Following release it is rapidly inactivated (Sigvardt et al. 1986). The α -BCPs are 100% identical in primary sequence across all Aplysiidae species so far examined (Li et al. 1999). The amino acid sequence is Ala-Pro-Arg-Leu-Arg-Phe-Tyr-Ser-Leu.

In *Lymnaea*, egg laying is also triggered by an array of neuropeptides, encoded by an ovulation hormone gene family, including the caudodorsal cell hormone (CDCH) I and II gene precursors (Vreugdenhil et al. 1988). These precursors produce at least nine caudo-dorsal cell peptides (CDCPs), including a 9 amino acid sequence encoding α -CDCP. Despite belonging to a different gastropod class to *Aplysia*, the α -CDCP retains the residues Arg-Leu-Arg-Phe, as encoded by the α -BCP gene. The CDCH precursor has a calculated molecular weight of 25 kDa, but due to a high percentage of charged amino acids, it migrates at 35 kDa in pulse-label experiments (Geraerts et al. 1985).

The first report of neuropeptide involvement in abalone reproduction was by Yahata (1973), who observed induced spawning of abalone after injection of homogenized ppg and visceral ganglia of mature females. Injections of homogenized cerebral ganglia (cg) produced no notable change in the ovaries. More recently, neurosecretion in the cg, ppg, and visceral ganglia was investigated in *H. discus hannai*, to determine the role of hormones in the regulation of reproduction (Hahn 1994). He found two types of cells in the cg that appeared to be neurosecretory. In another study involving the cg of *H. asinina*, two types of neurosecretory cells (NS), NS₁ and NS₂, were identified (Upatham et al. 1998). However, there is no experimental proof that the peptides produced in these cells regulate reproduction and growth.

A nucleotide sequence containing an abalone ELH (aELH) has been cloned from *H. rubra*, and shows high homology with the sequences of the CDCH of *Lymnaea* and ELH of *Aplysia* (Wang & Hanna 1998). However, no sequences have been obtained that encode an abalone α -CDCP-like peptide. Therefore, the current research work aimed to characterize α -CDCP-like immunoreactive structures within the abalone neural and reproductive systems to determine whether a homologous peptide was present.

*Corresponding author. E-mail: peter.hanna@deakin.edu.au

MATERIALS AND METHODS

Animals and Tissue Preparation

Mature male and female *H. rubra* (Leach) with ripe gonads were collected from Port Phillip Bay (Victoria) under Fisheries research permits (97/R/049A and RP 626). Tissues were then dissected for analyses of proteins, as well as preparation of tissue sections.

For protein extraction, approximately 200 mg of tissue was macerated, suspended in 2 mL SDS lysis buffer (2% SDS, 50 mM Tris-HCl (pH 7.2), 1 mM β -mercaptoethanol) and boiled for 3 min. The sample was homogenized (Industrial Equipment) and then boiled for a further 5 min. Protein solutions were microcentrifuged (Microcentaur MSE) at 13,000 rpm for 10 min and supernatant collected for storage at -70°C . Protein concentration was quantified using the Bradford method for protein quantitation (Kruger 1996).

Paraffin sections were prepared for immunocytochemistry by firstly fixing dissected tissues in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 h. They were then transferred to phosphate buffer and stored at 4°C before dehydration in an ascending ethyl alcohol series, xylene, then embedding in paraffin. Serial sections of 8 μm were cut with a microtome (Triangle Biomedical, CUT Series Rotary Microtome) and mounted on gelatin-coated slides. Paraffin sections of *H. discus hamai* ppg were kindly provided Dr. Kirk Hahn.

Antibodies Against *L. stagnalis* α -CDCP

Antiserum against α -CDCP peptide was kindly provided by Dr. Gregg Nagle (University of Texas Medical Branch, Galveston, Texas). This polyclonal antibody was made in rabbit and recognizes residues 144–152 (Glu-Pro-Arg-Leu-Arg-Phe-His-Asp-Val) of the *L. stagnalis* CDCH precursor (Vreugdenhil et al. 1988).

Western Blots

Larger proteins and peptides were separated by 12% SDS-PAGE using the method of Laemmli (1970), then electro-transferred by a Mini-Blot apparatus (Bio-Rad) to 45- μm nitrocellulose sheets (Bio-Rad). After transfer, the membrane was incubated with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS; 50 mM NaH_2PO_4 and Na_2HPO_4 , 154 mM NaCl, pH 7.4) for 2 h at room temperature (RT), with agitation. The membranes were incubated with the primary antibody, rabbit anti- α -CDCP, at a dilution of 1:1,000 (in Tris-buffered saline [TBS; 20 mM Tris-HCl pH 7.5, 500 mM NaCl] with 3% BSA) for 2 h. After several washes with PBS, the binding was visualized using HRP-coupled goat antirabbit antibody (Sigma), followed by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

Identification of small immunoreactive peptides was performed after 12% SDS PAGE (Gradipore) in Tris-Tricine buffer and subsequent electro-transfer to polyvinylidene fluoride (PVDF) sheets (Bio-Rad). Detection of α -CDCP-like peptides was performed as for the larger peptides.

Immunocytochemistry

Immunofluoresence was performed following dewaxing and rehydration of tissue sections. Blocking was then carried out, first in 1% glycine for 30 min, then in 4% BSA for 30 min. Sections were incubated for 1 h at RT in primary antibody (anti- α -CDCP) diluted 1:500 in PBS, rinsed three times in PBS, then incubated for

1 h at RT with FITC-labeled goat antirabbit secondary antibody (Sigma). Sections were rinsed three more times in PBS, and then mounted in FITC mounting solution (90% glycerol, 4% n-propyl-gallate in 50 mM PBS pH 8.2). The preparations were examined under a fluorescence microscope (Leica) and the images were captured with a Spot cooled CCD camera (Diagnostic Instruments).

Immunoenzyme staining was performed following dewaxing and rehydration of tissue sections. Endogenous peroxidase activity was quenched by incubation of sections in 3% H_2O_2 in methanol for 10 min. After washing in PBS, blocking was performed, first in 1% gelatin for 30 min then in 4% BSA for 30 min. Sections were rinsed three times in PBS, incubated for 1 h at RT in primary antibody diluted 1:1000 in PBS, then rinsed three times in PBS, and incubated in HRP-labeled goat antirabbit secondary antibody (Sigma) in the dark at RT. After three rinses in PBS, the sections were developed in 3-amino-9-ethylcarbazole (AEC) substrate solution (ICN). When sufficient color occurred, development was stopped by a wash in distilled water, and the sections counter-stained in Mayer's haematoxylin. After a further wash the sections were mounted in Faramount aqueous mounting solution (DAKO) and viewed under a Zeiss Axioskop MC 80 microscope. Images were captured on a Spot cooled CCD camera (Diagnostic Instruments).

Controls

For each experiment it was important to perform controls to ensure antibody specificity. Immunoreactivity of the respective peptides was not observed following antibody preincubation with crude abalone tissue protein (data not shown). Also, no immunoreactivity was observed when blots or sections were incubated with no primary antibody (data not shown).

RESULTS

Immunoblotting

Figure 1A shows the results of Western blots of combined cg and ppg tissues, mature male gonad (mgo), and mature female gonad (fgo) sample separations transferred to nitrocellulose and reacted with anti- α -CDCP as the primary antibody. In the combined cg-ppg sample, a protein was identified of approximately 100 kDa. The mature mgo and fgo also contained the 100 kDa protein, but also contained immunoreactive proteins approximately 48 kDa and 46 kDa in size.

Additional smaller immunoreactive proteins were identified by chemiluminescence detection in Western blots performed using SDS-PAGE in Tris-Tricine buffer and transfer to PVDF membranes (Fig. 1B). The combined cg-ppg sample now showed the presence of the 48 kDa and 46 kDa proteins, as well as additional smaller ones of approximately 36 kDa and 29 kDa. The mgo contained additional immunoreactive 32 kDa and 22 kDa proteins, whereas the fgo contained additional 36 kDa, 28 kDa, and 23 kDa proteins.

Western blots of fractionated total proteins isolated from the *H. rubra* heart, foot, rectum, gill, and tentacle also showed a 100 kDa protein that was immunoreactive with anti- α -CDCP (Fig. 1C). As well, the 48 and 46 kDa proteins were also present in all tissues, except the foot tissue.

Immunocytochemistry

Immunocytochemistry studies, using an anti- α -CDCP probe on sections of neural and gonadal tissues, showed strong positive

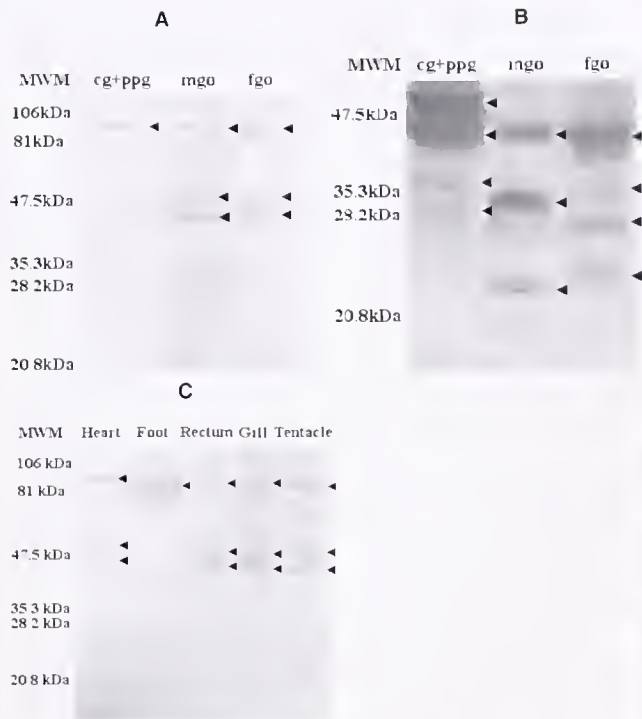


Figure 1. Western blots of *H. rubra* tissues using an anti- α -CDCP. Total proteins were isolated from *H. rubra* tissues then separated by 12% SDS-PAGE with Tris-Glycine buffer or 12% SDS-PAGE with Tris-Tricine buffer, and electro-transferred to nitrocellulose or PVDF, respectively. After blocking, the membranes were then reacted with rabbit anti- α -CDCP, followed by HRP-conjugated antirabbit Ig, and detection was achieved using ECL reagents (Amersham). Arrows show localization of immunoreactive proteins. MWM, prestained molecular weight markers (Bio-Rad). **A.** Immunodetection of 100 kDa in the combined cg and ppg tissues, and 100, 48, and 46 kDa proteins in the mgo and fgo (SDS-PAGE then nitrocellulose). **B.** Immunodetection by chemiluminescence showing additional 48, 46, 36, and 29 kDa proteins in the combined cg and ppg tissues, 32 and 22 kDa proteins in the mgo, and 36, 28, and 23 kDa proteins in the fgo (SDS-PAGE then PVDF). **C.** Immunodetection of a 100 kDa protein in total protein extracts of heart, foot muscle, rectum, gill, and tentacle, as well as 48 and 46 kDa proteins in all tissues, except the foot muscle (SDS-PAGE-nitrocellulose).

results (Figs. 2A to H). The cg and ppg of the mature *H. rubra* contained numerous immunopositive cells (Figs. 2A to F). Immunoreactive cells of the cg were distributed throughout the cortex, but were mainly located within the outer cells and within the inner cortex (Figs. 2A and B). The outer cells appear to be the neurosecretory NS₁ and NS₂ cells identified by Upatham and colleagues (1998). A large number of immunoreactive axons were found to extend into the medulla of the left and right cg, and through the dorsal cerebral commissure.

The ppg also contained abundant immunopositive cells that were distributed within the cortex region (Figs. 2C and D). Many of these cells were likely to be of the NS type because they were located close to the basement membrane. In addition, the immunoreactivity was strong, indicating there was a large amount of reactive protein present. A high density of immunoreactive axons was identified transversing the medial and lateral regions of the medulla in the ganglion mass, and extending into the pedal and cerebro-peleural connectives (Figs. 2E and F). No immunoreactiv-

ity was observed within the statocysts. As well, no difference was observed in peptide distribution in the neural tissues between female or male *H. rubra*.

To show conservation between abalone species, ppg sections prepared from *H. discus hannai* were also tested with anti- α -CDCP. Numerous immunopositive fibers were present in the medulla of the ppg of *H. discus hannai* (Fig. 2G). No reactivity was seen in the cortex, possibly due to Stieve's fixative used to prepare these ganglia, and the antibodies may not have been able to penetrate cortex cells, the antigen destroyed by fixation, or the peptide was not present in these cells. However, as observed in *H. rubra*,

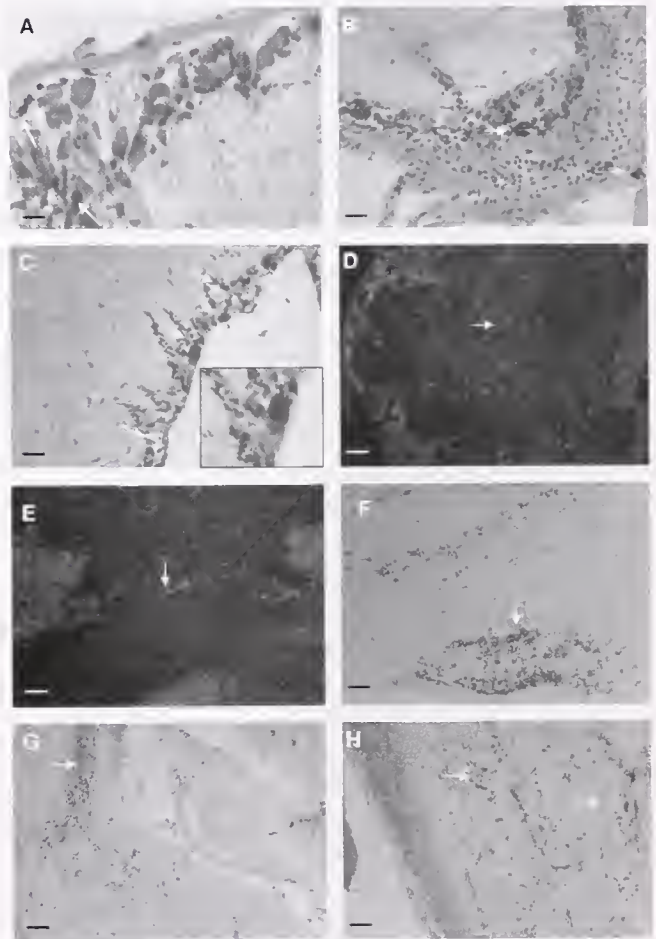


Figure 2. Immunocytochemical localization of an α -CDCP-like peptide in *H. rubra* and *H. discus hannai* neural tissues. Sections were first incubated with rabbit anti- α -CDCP, prior to detection with HRP-conjugated antirabbit Ig and an AEC substrate to produce a red color, or immunofluorescence using FITC-conjugated antirabbit Ig. Sections were counterstained with hematoxylin. **A** and **B.** *H. rubra* sections showing immunoreactivity in the cortex cells (arrows) of a cg and axons (arrowhead) extending into the medulla (bars 18.75 and 25 μ m, respectively). **C.** *H. rubra* section showing immunoreactivity in cortex cells (arrows) of a ppg (bar 25 μ m). Inset is a high power micrograph showing abundant staining (arrowhead) of a cell at the basement membrane. **D** and **E.** Immunofluorescence in *H. rubra* axons located in the medulla of a ppg (bars 25 μ m). **F.** Immunopositive reactivity in axons that extend laterally into the pedal ganglia from the ppg (bar 25 μ m). **G** and **H.** *H. discus hannai* immunopositive axons (arrows) in the medulla of a ppg. Asterisk indicates the area of an immunoreactive loop (bars 50 μ m and 25 μ m, respectively).

intensely stained axons were observed throughout the medulla, and appeared to form a loop (Fig. 2H).

Immunoreactive material to anti- α -CDCP was localized in the fgo and mgo of *H. rubra* (Fig. 3A to C). In the fgo, immunopositive protein was demonstrated in special cells and fibers of the trabeculae (Fig. 3A). In addition, a small number of immunoreactive cells and fibers were identified within the gonad capsule, and the hepatopancreas capsule (Fig. 3B), but never within the oocyte nucleus. Control sections corresponding to the same tissue resulted in no positive signals. The level of immunoreactivity in the gonads appeared to differ between individual animals, possibly as a result

of oocyte maturation. In the mgo, immunoreactivity was restricted to cells and fibers of the trabeculae (Fig. 3C), as observed in fgo. Also, a small number of immunoreactive cells were located within the gonad capsule and hepatopancreas capsule (data not shown).

DISCUSSION

The focus of our research involves the eg, ppg, and gonad tissues, due to their importance in gastropod reproduction (Geraerts et al. 1988, Yahata 1973). The difficulty associated with the isolation of the visceral ganglia hindered any experimental analysis on this tissue.

The expression of an α -CDCP-like peptide in abalone was firstly identified by Western blots. Total protein isolated from abalone tissues confirmed that the anti- α -CDCP probe was reactive to a protein of 100 kDa in the combined eg and ppg tissues, as well as the gonads of *H. rubra*. Reactive peptides of 46 and 48 kDa were also detected in the gonad tissue. This finding was consistent with peptides being synthesized from a 100-kDa precursor protein, but was larger than the 32 kDa identified in *Aplysia* (Berry 1981), or 25 kDa as observed in *Lymnaea* (Geraerts et al. 1985).

Due to the small size of a fully processed α -CDCP (9 aminoacids), it was expected that a homologous α -CDCP-like peptide in abalone would not be detected in standard blots. However, a number of possible intermediates were observed and is consistent with the findings in *A. californica*, that post-translational processing by specific convertases produces peptides of different sizes and these can be post-translationally modified (Fisher et al. 1988). They showed by Western blot analysis that antibodies recognized small final-product peptides and intermediates in the processing pathway, but all contained the sequence encoding the immunogen. Thus, the different sized bands observed in *H. rubra* Western blots probably represent differential cell processing of the polypeptides. Our results indicate that the abalone precursor of 100 kDa is initially processed to release a 35-kDa protein (aELH immunoreactive, Cummins et al. 2002) and a 48-kDa protein (α -CDCP immunoreactive, this study), prior to further processing. If this is correct, the size of the precursor is much larger than that shown for the *Aplysia* ELH egg-laying precursor peptide (32 kDa), and may indicate that the genetic make-up of the *H. rubra* reproductive precursor is different to *Aplysia* and *Lymnaea* homologues. However, the multiple bands may also be the result of expression of a multigene aELH family, as shown in *Aplysia* species (Scheller et al. 1983), in which different expression occurs in different tissues.

Western blots also showed the α -CDCP-like peptide distribution in the heart, foot muscle, rectum, gill, and tentacle tissue, suggesting that these peptides may have a broad function in this animal. Similarly, peripheral tissues in *L. stagnalis* are known to contain immunoreactive material to egg-laying peptides (Van Minnen et al. 1989). However, subsequent immunohistochemical analyses in this laboratory have shown that immunoreactivity is predominantly localized to neural ganglia, therefore the other positive material may only be an indication of transport of protein or peptides.

Previous immunocytochemical studies have successfully used antibodies against *Aplysia* and *Lymnaea* egg-laying peptides to demonstrate their presence in the neural systems of other invertebrates. For example, peptides homologous to ELH have been immunolocalized in the neural cells of *Busycon* and *Mytilus* (Ram et al. 1998, Croll et al. 1993). Also, α -CDCP is expressed in the

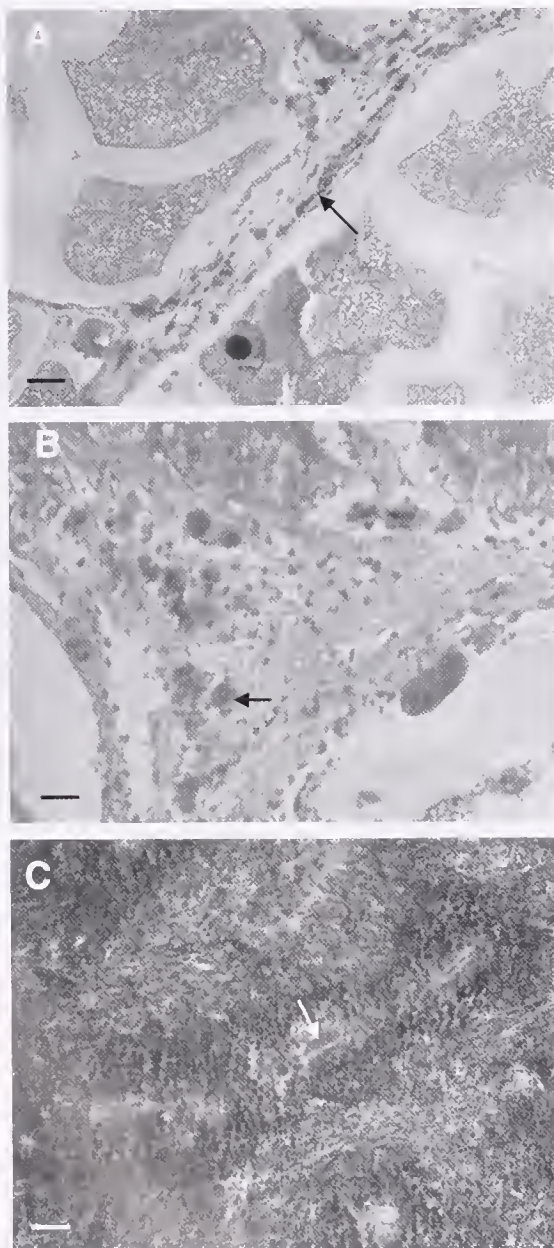


Figure 3. Immunocytochemical localization (arrows) of an α -CDCP-like peptide in *H. rubra* gonad and hepatopancreas tissues. Sections were treated as in Fig. 2. A. Immunoreactivity in cells of the fgo trabeculae (bar 25 μ m). B. Immunoreactivity in cells of the hepatopancreas capsule of a fgo (bar 27.5 μ m). C. Immunoreactivity in cells of the mgo trabeculae (bar 25 μ m).

neural network of *Helix aspersa*, *Mytilus edulis*, and *Busycon canaliculatum* (Croll et al. 1993, Ram et al. 1998). In a previous study, peptides immunoreactive to antisera against CDCH, α -CDCH and β -CDCH, showed positive immunoreactions in tissues of *Sarcophaga bullata* (Diptera), *Leptinotarsa decemlineata* (Coleoptera), *Locusta migratoria*, and *Periplaneta americana* (Orthoptera) (Theunis et al. 1990). More recently, it has been shown that antisera directed against the same three peptides results have been detected in the central nervous system of the rhyonohdellid leech, *Theromyzon tessulatum* (Salzet et al. 1997).

Our use of anti- α -CDCP in immunocytochemistry has demonstrated that an α -CDCP-like peptide is extensively distributed throughout *H. rubra* cg and ppg tissues. These results imply that the cg could be important in the regulation of reproduction in gastropods. The same distribution pattern was observed for aELH (Cummins et al. 2002), and enhances the possibility that these peptides are translated as a single precursor. Immunoreactivity appeared to be localized to the NS₁ or NS₂-type cells in the cortex. However, it seemed that the distribution of immunoreactive cells containing aELH and α -CDCP-like peptide were not concentrated within one specific region or cluster of cells, but they were widely distributed within the cortex cells and axons. These reproductive peptides may be released from much of the surface of the nervous system and not just from well-defined neurohemal organs. This is unlike studies of other molluscs, in which antisera have been shown to react with specific clusters of neuronal populations in the ganglia (i.e., such as the immunoreactivity pattern of antiCDCH in *L. stagnalis* (Van Minnen et al. 1988). However, we did observe axonal loops within the medulla of ganglia by anti- α -CDCP immunolocalization. It has been suggested that similar loops in *L. stagnalis* are important sites of synaptic integration (Van Minnen et al. 1988).

Concerning the function of the immunoreactive neurons, it is possible that reproductive peptide colocalization may facilitate their simultaneous release into the surrounding hemolymph, as is the case in other gastropods (Chiu & Strumwasser 1981, Bernheim & Mayeri 1995). Extensive immunoreactive axonal distribution throughout the medulla of the cg and ppg suggests that these may lead to sites of neuropeptide release. In *Lymnaea*, the CDCs release egg-laying peptides into the hemolymph via neurohemal areas and also from blind ending axons in the medulla of the commissure (Van Minnen et al. 1989). We can also speculate that, similar to *Aplysia* and *Lymnaea*, *H. rubra* reproductive peptides too may control reproduction through autoexcitation or autoinhi-

bition of other neural tissues. However, to fully elucidate their biologic activity, further experiments involving the identification of a precursor sequence, bioassays and electrophysiology would be required. Given that abalone are broadcast spawners (not internal fertilizers), peptide function will invariably be different to *Aplysia* and *Lymnaea*. Further investigations are also required to provide a more detailed analysis of the cellular structure of the immunoreactive cells. Although our study using light microscopy predicts that they are of NS type, more definitive ultrastructural studies would provide more precise details.

Despite extensive research being conducted on neuropeptide immunoreactivity within the central nervous system, relatively little research has focused on the reproductive tissue. However, CDCH genes have been immunocytochemically localized to the female part of the reproductive tract and exocrine secretory cells of the male part (Van Minnen & Vreugdenhil 1987). Our study has shown that an α -CDCP-like peptide is present in the trabeculae of the fgo and in mgo trabeculae of *H. rubra*. The peptide was only located within certain cell types, which may function as a peptide storage cell for the maturing gonad. It has been suggested that the granulated cells of the gonad trabeculae are the endocrine cells of the gonads (Apisawetakan et al. 2001). Indeed, recent immunocytochemical experiments performed using antiELH on *H. asinina* gonad tissues, have shown high immunoreactivity in the granulated cells (Chanpoo et al. 2001). The large amount of reproductive-like peptides within the fgo may be a function of the gastropod requirement to release high concentrations of hormone into the hemolymph or gonad to cause maturation of oocytes prior to spawning. It is known in molluscs, that hormone release areas are high in number (Joosse 1988).

The observation of reproductive peptides in the male *H. rubra* trabeculae is not unusual. Evidence from *Drosophila melanogaster* shows that when a male mates and releases sperm, he also deposits an ELH that induces egg laying in the female (Park & Wolfner 1995). This hormone is related, by use of cross-reactive antisera, to the ones found in gastropods and other invertebrates. In addition, *L. stagnalis* CDCH gene products are known to be secreted into the male duct and transferred to the female copulant during copulation, and may function to accelerate the start of egg laying (Van Minnen et al. 1989). Thus, *H. rubra* trabeculae reproductive peptides may play a similar role, but because they are spawners, this role may be altered.

In summary, these results indicate that α -CDCP-like peptide could play a significant role in abalone reproduction.

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GENETIC STRUCTURE OF CULTURED *HALIOTIS DIVERSICOLOR SUPERTEXTA* (REEVE) POPULATIONS

ZHONGBAO LI^{1,2*} AND CHANGSHENG CHEN¹

¹Fisheries College, Jinxi University, Xiamen, Fujian 361021, People's Republic of China; ²School of Marine and Environmental Studies, Xiamen University, Xiamen, Fujian 361005, People's Republic of China

ABSTRACT Genetic structure of four cultured *Haliotis diversicolor supertexta* populations were investigated using the assay of vertical slab polyacrylamide gel electrophoresis. Low levels of genetic diversity of populations was found: mean numbers of alleles per locus ranged from 1.4 to 1.5; proportions of polymorphic loci ($P_{0.99}\%$) ranged from 27.78 to 33.33; observed heterozygosities ranged from 0.120 to 0.150; expected heterozygosities ranged from 0.123 to 0.131. The results showed that the coefficient of gene differentiation among populations was low ($F_{ST} = 0.04$), gene flow among populations was large ($Nm = 6$). Genetic structure was very similar among four cultured populations. The genetic distances between any two of the four populations were 0.002–0.009, with an average of 0.0047.

KEY WORDS: *Haliotis diversicolor supertexta*, allozyme, genetic structure, genetic diversity, genetic differentiation

INTRODUCTION

Haliotis diversicolor supertexta (Reeve) is widely distributed in China and Japan (Nie & Wang 2000). In recent years, *H. diversicolor supertexta* has been playing an economically important role in the fishing and farming industries of China. Numerous studies on the culture and ecology have been undertaken on *H. diversicolor supertexta*, however, few are concerned with genetic structure. Knowledge of genetic structure among populations is particularly important for managing exploited species. This knowledge is used in aquaculture to assess the potential for adaptive divergence available from different sources of broodstock. To protect this valuable marine resource and supply genetic information for the study of genetics and breeding, it is necessary to investigate and assess the genetic structure of *H. diversicolor supertexta* population.

Allozyme electrophoresis is considered to be an extremely useful technique in population genetics (Allendorf & Utter 1979, Lavery & Shaklee 1991). Within the genus *Haliotis* this kind of study has only been carried out in *H. fulgens* (Zúñiga et al. 2000), *H. roei* Grey (Hancock 2000), *H. rubra* Leach (Brown 1991, Brown & Murray 1992) and *H. laevigata* Donovan (Brown & Murray 1992). This technique is used in the present study to examine the genetic structure of cultured *H. diversicolor supertexta* populations. In this study we describe the pattern of population genetic structure in four cultured *H. diversicolor supertexta* populations from one commercial abalone farm of Dongshan (population 1) and three commercial abalone farms of Zhangpu (population 2, population 3, and population 4) in Fujian Province, using allozyme analysis. The results can be useful for protecting and improving the resources of this species.

MATERIALS AND METHODS

Samples

Vertical slab polyacrylamide gel electrophoresis was used to estimate genetic structures and genetic diversities of the four cultured *H. diversicolor supertexta* populations. One hundred and twenty eight individuals were collected in 2002 from one commercial abalone farm in Dongshan (population 1, 32 individuals)

and three commercial abalone farms in Zhangpu (population 2, 32 individuals; population 3, 32 individuals; and population 4, 32 individuals) in Fujian Province. They were alive on the way to the laboratory. A muscle tissues was dissected from each individual and preserved in a deep freezer at -80°C . The muscle sample (0.5 g) was homogenized with Tris-HCl extract solution ($\text{pH} = 7.0$). The homogenates were centrifuged at 12000 rpm for 15 min at 4°C . The supernatant was then collected for electrophoresis.

Electrophoresis

The allozymes were detected using polyacrylamide gel electrophoresis (PAGE). The buffer systems and staining procedure followed those described by Taniguchi and Sugama (1990), Zen and Xiang (1989), and Wang (1996). A total of 10 enzymes at 18 loci were analyzed: lactate dehydrogenase (LDH, E.C.1.1.1.27), superoxide dismutase (SOD, E.C.1.15.1.1), esterase (EST, E.C.3.1.1.-), aspartate aminotransferase (AAT, E.C.2.6.1.1), alcohol dehydrogenase (ADH, E.C.1.1.1.-), malic enzyme (ME, E.C.1.1.1.40), malate dehydrogenase (MDH, E.C.1.1.1.37), sorbitol dehydrogenase (SDH, E.C.1.1.1.14), isocitrate dehydrogenase (IDH, E.C.1.1.1.42), and amylase (AMY, 3.2.1.1). The names, abbreviations and numbers of the enzymes were described by Shaklee et al. (1990), and the description of the loci and alleles by Wang (1996).

Statistical Analysis

To assess the genetic diversities of the four populations, the allele frequencies, the mean numbers of alleles per locus (A), the proportions of polymorphic loci (P), observed heterozygosities (H_o), and expected heterozygosities (H_e) at Hardy-Weinberg equilibrium were calculated (Wang 1996). A locus was considered to be polymorphic ($P_{0.99}\%$) if the frequency of most common allele was equal or less than 0.99 at one or more localities. To estimate the degree of genetic differentiation among populations, the genetic distances between any two of the four populations were calculated using Nei's formulas (Nei 1978), the coefficient of gene differentiation (F_{ST}) among populations was calculated following Wright (1977), and gene flow among populations (Nm) was calculated using Wright's formulas (Wright 1969). Dendrogram from the matrix of genetic distances was constructed using unweighted pair group method with arithmetic averaging (UPGMA, Sneath & Sokal 1973). Data were analyzed using BIOSYS-1 software (Swofford & Selander 1989).

*Corresponding author. Fax: +86-592-618-1420; E-mail: zhongbaoli@hotmail.com

TABLE 1.

Allele frequencies at polymorphic loci in cultured *Haliotis diversicolor supertexta* populations.

Locus	Allele	Population 1	Population 2	Population 3	Population 4
<i>Est-2</i>	A	0.333	0.438	0.708	0.646
	B	0.667	0.563	0.292	0.354
<i>Est-3</i>	A	0.854	0.792	0.875	0.708
	B	0.125	0.208	0.125	0.229
	C	0.021	0.000	0.000	0.063
<i>Mdh-1</i>	A	0.188	0.188	0.188	0.167
	B	0.813	0.813	0.813	0.833
<i>Sdh-2</i>	A	0.979	1.000	1.000	1.000
	B	0.021	0.000	0.000	0.000
<i>Aat-1</i>	A	0.042	0.021	0.167	0.042
	B	0.188	0.146	0.292	0.146
	C	0.313	0.354	0.208	0.354
	D	0.458	0.479	0.333	0.458
<i>Amy-1</i>	A	0.438	0.688	0.563	0.417
	B	0.563	0.313	0.438	0.583

RESULTS

Ten enzymes coded by 18 loci were clearly resolved in all populations (Li et al. 2004a). Six loci (*Est-2*, *Est-3*, *Mdh-1*, *Sdh-2*, *Aat-1*, *Amy-1*) were polymorphic in at least one population among populations. Allele frequencies at polymorphic loci in four cultured *H. diversicolor supertexta* populations are presented in Table 1 and showed little variability among them.

Low levels of genetic diversity were estimated in the four cultured *H. diversicolor supertexta* populations (Table 2). The mean numbers of alleles per locus ranged from 1.4 to 1.5. The proportions of polymorphic loci ($P_{0.99\%}$) ranged from 27.78 to 33.33. Observed heterozygosities ranged from 0.120 to 0.150. Expected heterozygosities ranged from 0.123 to 0.131. The genetic diversity was very similar among populations.

To estimate the degree of genetic difference among the four populations, the coefficient of gene differentiation (F_{ST}) and the genetic distance (D) were calculated. The coefficient of gene differentiation among populations was 0.04 (Table 3). The genetic distances between any two of the four populations were 0.002–0.009, with the average of 0.0047, the genetic identities between any two of the four populations were 0.991–0.998, with the average of 0.9953 (Table 4). Gene flow was large ($Nm = 6$) (Table 3). The UPGMA dendrogram of four cultured *H. diversicolor supertexta* populations was shown in Figure 1. The first formed by population 1 and population 2 at a distance of 0.002. The second

TABLE 2.

Genetic diversities of cultured *Haliotis diversicolor supertexta* populations.

Index	Population 1	Population 2	Population 3	Population 4
A	1.5 (0.2)	1.4 (0.2)	1.4 (0.2)	1.4 (0.2)
$P_{0.99\%}$	33.33	27.78	27.78	27.78
H_o	0.134 (0.068)	0.137 (0.063)	0.120 (0.060)	0.150 (0.069)
H_e	0.124 (0.051)	0.124 (0.051)	0.123 (0.053)	0.131 (0.053)

TABLE 3.

F-statistics and gene flow at polymorphic loci in cultured *Haliotis diversicolor supertexta* populations.

Locus	F_{IS}	F_{IT}	F_{ST}	Nm
<i>Est-2</i>	0.493	0.540	0.093	
<i>Est-3</i>	0.029	0.051	0.022	
<i>Mdh-1</i>	-0.084	-0.083	0.001	
<i>Sdh-2</i>	-0.021	-0.005	0.016	
<i>Aat-1</i>	-0.508	-0.477	0.020	
<i>Amy-1</i>	-0.206	-0.149	0.047	
Mean	-0.130	-0.060	0.040	6

formed by population 3 and population 4 ($D = 0.002$), which was linked to the first cluster at a distance of 0.006.

DISCUSSION

Genetic diversity is fundamental for the maintenance of species, population, and ecosystem diversities. Many studies have demonstrated that a loss of genetic diversity leads to a reduction in the adaptive fitness of a population, increasing the risk of extinction (Vrijenhoek 1994). In the present study, low level of genetic diversity was estimated in the four cultured *H. diversicolor supertexta* populations (Table 2). The proportions of polymorphic loci of *H. diversicolor supertexta* populations ranged from 27.78 to 33.33, which was lower than that of 48 kinds of seashells ($P = 0.471 \pm 0.159$) (Singh 1984). The proportion of polymorphic loci is influenced by the number of loci examined. The average observed heterozygosity we found among *H. diversicolor supertexta* populations ($H_o = 0.135$) was similar to those reported for *H. rubra* ($H_o = 0.136$; Brown 1991), *H. fulgens* ($H_o = 0.119$; Zúñiga et al. 2000), *H. discus hannai* Ito ($H_o = 0.123$; Fujio et al. 1983) and 48 kinds of seashells ($H_o = 0.147$; Singh & Green 1984), however, lower than that of *H. laevigata* ($H_o = 0.195$; Brown & Murray 1992) and higher than that of 10 kinds of invertebrates ($H_o = 0.098$; Powell 1975). It is probably a reasonable assumption that the amount of isozyme variation reflects the relative amount of genetic variation found at other loci in the genome (McAndrew & Majumder 1983). Six loci (*Est-2*, *Est-3*, *Mdh-1*, *Sdh-2*, *Aat-1* and *Amy-1*) in 18 gene loci were polymorphic in at least one population among populations. From the genetic point of view, *Est-2*, *Est-3*, *Mdh-1*, *Sdh-2*, *Aat-1* and *Amy-1* can be used as the biochemical genetic markers of *H. diversicolor supertexta* for genetic diversity assessment and selective breeding program. Similar studies have been carried out in *H. diversicolor diversicolor* (Li et al. 2004a), *H. discus discus*, and *H. discus hannai* (Li et al. 2004b).

TABLE 4.

Genetic distances (above diagonal) and genetic identities (below diagonal) among cultured *Haliotis diversicolor supertexta* populations.

Population	1	2	3	4
1	*****	0.002	0.009	0.004
2	0.998	*****	0.006	0.005
3	0.991	0.994	*****	0.002
4	0.996	0.995	0.998	*****

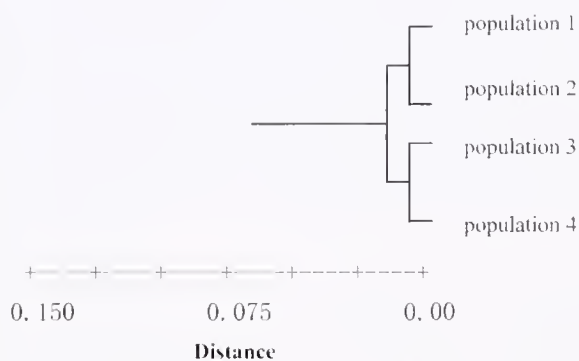


Figure 1. The unweighted pair group method with arithmetic averaging (UPGMA) of the four cultured *Haliotis Diversicolor supertexta* populations.

Genetic structure was very similar among four cultured populations. The coefficient of gene differentiation among populations was low ($F_{ST} = 0.04$), with 4% gene differentiation of the total diversity coming from interpopulation and 96% from intrapopulation. The result showed that gene flow among populations in our study was large ($Nm = 6$) (Table 3). The estimate obtained in this study for F_{ST} values in *H. diversicolor supertexta* is in general agreement with those reported for *H. cracherodii* ($F_{ST} = 0.039$; Hamm & Burton 2000), *H. fulgens* ($F_{ST} = 0.036$; Zúñiga et al.

2000), *H. rubra* ($F_{ST} = 0.022$; Brown 1991), *H. laevigata* ($F_{ST} = 0.014$; Brown & Murray 1992), *H. rufescens* ($F_{ST} = 0.012$; Burton & Tegner 2000) and *H. roei* ($F_{ST} = 0.009$; Hancock 2000) within the genus *Haliotis*.

The mean genetic distance and mean genetic identity among populations were 0.0047 and 0.9953 respectively, which means that there were no significant differences in the biochemical genetic character among populations. Nei (1987) considers the threshold genetic distance between populations, between subspecies, and between species at about 0.01, 0.1, and 1.0. They belong to population level of *H. diversicolor supertexta*.

Modern fisheries science demands a holistic management of fisheries; this concept includes the maintenance of genetic diversity and population structure, which are critical for ensuring the long-term survival of any fishery (Shepherd & Brown 1993). The results in this study are likely to be indicative of population genetics of cultured *H. diversicolor supertexta* in China and the factors influencing it, which should be considered in the development of *H. diversicolor supertexta* conservation and future management plans.

ACKNOWLEDGMENTS

The project was supported by National Natural Science Foundation of China (No:30271013), Natural Science Foundation of Fujian province (No:B0110036), and The project of the Committee of Science and Technology of Fujian Province (No:2001Z073).

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RAPD ANALYSIS OF GENETIC DIVERSITIES OF THREE SPECIES OF ABALONE

TAIWU LI,¹ WENXIN YANG,² XIURONG SU,¹ ZHIBIAO YANG,¹ AND HAO GUO³

¹Faculty of Life Science and Biotechnology, Ningbo University, Ningbo, 315211, China; ²Life Science College, Liaoning Normal University, Dalian, 116029, China; ³National Marine Environment Monitoring Center, Dalian, 116023, China

ABSTRACT RAPD (random amplified polymorphic DNA) technique was applied to detect the genetic diversity in 3 species of abalone (*Haliotis discus hannai* Ino, *Haliotis discus discus* Reeve and *Haliotis diversicolor* Reeve). Twenty random primers, screened out of 100, gave 213 clear and informative fragments, ranging from 250–2600 bp, under predetermined optimal reaction conditions. There existed genetic diversity not only between species but also among individuals. The percentage of polymorphic loci, genetic heterozygosity, genetic identity, and genetic distance were calculated to reveal the diversities among species by using the Popgene32 software and a phylogenetic tree was constructed with the method of UPGMA. The results indicated that genetic variation in the population of *H. discus discus* is relatively high. *H. discus hannai* and *H. discus discus* showed a very close relationship with each other but a relatively distant relationship with *H. diversicolor*.

KEY WORDS: abalone, RAPD, genetic diversity

INTRODUCTION

RAPD marker can detect the insertion, loss of the binding sites distributed throughout the target genome with the differences of the amplified fragments. Compared with other molecular markers, such as RFLP, RAPD marker is simpler, lower time- and money-consuming and requires less DNA template, and doesn't need prior knowledge of DNA sequence. It has been widely used in genetic variability analysis, identification of relationship and genetic breeding and so on (Liu & Xiang 1996, Liu et al. 1996, Li & Zou 1999, Zhang et al. 2000) since it was established in 1990 (Williams et al. 1990, Welsh & McCland 1990). In recent years, there were some successful reports in China and elsewhere, such as American system project of HHS (high health shrimp), oyster (Liu & Dai 1998), and shrimp (Song et al. 1998, 1999).

Abalone belongs to Mollusca, Gastropoda, Prosobranchia, Haliotidae, *Haliotis*. It is a precious marine shellfish with great value both as a health food and medicine, living on rocks and reefs at about 10 m depth from the low water mark. It is reported that there are seven species of abalone in China. Two of them have high economic value, namely *Haliotis discus hannai* Ino and *Haliotis diversicolor* Reeve. The former lives in the northern sea of China, Japan and Korea (Hou 1998), with that from Japan being regarded as a geographical subspecies of *Haliotis discus hannai*, named *Haliotis discus discus*. The latter is a warm sea species distributed in Fujian, Taiwan, Guangdong of China, and the south part of Japan. Previous studies mostly focused on ultrastructure (Liu et al. 2000, Ke et al. 2003, Cui et al. 2004) and diseases diagnosis (Li et al. 1998, Chen et al. 2000), but there are few systematic studies on the genetic diversity of abalone in China. In the present study, the RAPD technique was applied to assess the genetic diversity and the relative relationship of the three species of abalone to provide some potential theoretical reference for ecological protection and reasonable exploration of the marine resources.

MATERIALS AND METHODS

Samples

Twelve *Haliotis discus hannai* Ino were collected from Zhangzi Island of Dalian, Liaoning Province (named population Z), and nine cultured *Haliotis discus discus* provided by the Key Laboratory of Marine Bioengineering in Ningbo University, Zhejiang Province (population B), and 20 cultured *Haliotis diversicolor* Reeve from Xiamen, Fujian province sea area (population F).

Random Primers

Ten-Mer random primers were bought from Shenggong Biotechnology Ltd. Shanghai Co., 20 primers were screened from 100, using 1 animal from each group (Table 1).

Instruments & Reagents

Amplifications were carried out on the Gene Amp PCR system made by PERKIN ELMER Analyze Co. in US. Cary 100 Cone U-visible Spectrophotometer was used to test the purity and concentration of DNA. FR-980 biologic electrophoresis test system was used to take photographs of gels.

Proteinase-k, Taq DNA polymerase, and dNTP were the products of TaKaRa Co. The other A.R grade reagents were bought from local Chemical Reagents Co.

DNA Extraction

One hundred milligrams of abdominal foot muscle fixed with alcohol was incubated at 55 °C for about 4 h in microcentrifuge tubes containing TEN (10 mM Tris-HCl, Na₂-EDTA, 0.15 M NaCl, pH 8.3), 2% SDS and 200 µg Proteinase-K, then extracted with phenol/Chloroform (1:1), and then Chloroform: isopentanol (24:1). The supernatant fractions were mixed with two volumes of cold 100% alcohol and 1/10 volume of 3 M sodium acetate. The DNA quality was tested by running the samples in 0.7% agarose gel containing ethidium bromide (Fig. 1). Its purity and concentration were tested with Cary 100 Cone system (Li et al. 2003).

PCR Amplification

PCR amplification was performed in a 25 µL reaction volume containing 40 ng genomic DNA, 0.2 mM dNTP at 200 µM final

*Corresponding author. E-mail: litaiwu@hotmail.com

TABLE 1.
Random primer sequence.

Primer	Sequence	Primer	Sequence
S01	5'-GTTTCGCTCC-3'	S18	5'-CCACAGCAGT-3'
S02	TGATCCCTGG	S20	GGACCCTTAC
S04	GGACTGGAGT	S21	CAGGCCCTTC
S05	TGCGCCCTTC	S22	TGCCGAGCTG
S06	TGCTCTACCC	S41	ACCGCGAAGG
S12	CCTTGACGCA	S46	ACCTGAACGG
S13	TCCCCCGCT	S55	CATCCGTGCT
S14	TCCGCTCTGG	S60	ACCCGGTCAC
S15	GGAGGGTGTT	S73	AAGCCTCGTC
S17	AGGGAACGAG	S81	CTACGGAGGA

concentration, 2.5 μ L 10 \times PCR buffer, 40 ng primer, 2 mM $MgCl_2$, 2.0 U of Taq DNA polymerase and ddH₂O. Amplification was carried out in the Gene Amp PCR System at predenaturation at 94 °C for 5 min, then total 40 cycles were run as follows: denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min, extension at 72 °C for 1 min. After the final cycle, reaction mixtures were incubated for a further extension at 72 °C 10 min. The amplification products were run in 1.3% agarose gel containing ethidium bromide (0.3 μ g/mL) in TBE buffer (89 mmol/L Tris-HCl, 89 mM boric acid, 10 mM EDTA, pH 8.3). The gels were electrophoresed at 40 V for ~2 h and photographed.

Data Analysis

PCR amplified DNA bands were recorded as 1 for presence and 0 for absence. Using the "0, 1" matrices, the percentage of polymorphic loci (P), average genetic heterozygosity (H) and genetic distance (D) of three species of abalone were calculated using Popgen32 software:

$P = \text{polymorphic fragments} / \text{total amplified fragments}$

$D = -\ln^{1/2} [J_{xy} / (J_x J_y)^{1/2}]$ (Nei & Li 1979). J_{xy} is the average genetic identity between population x and y. J_x and J_y are the average genetic identity of population x and y respectively.

$$H = \sum_{i=1}^n (1 - \sum X_i^2) / n$$

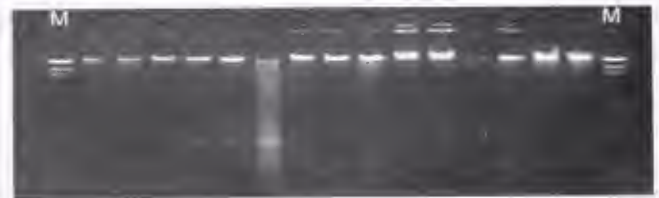


Figure 1. Electrophoresis patterns of DNA. M: λ DNA/Hind III.

(Nei 1978). x_i is the frequency of allele X_i , n is the number of tested loci.

Cluster analysis is based on D with the UPGMA (unweighted pair group method using arithmetic mean).

RESULTS

RAPD Analysis

The screened 20 primers yielded 213 bands ranging from 250–2600 bp and most of them were 500–2000 bp. Each primer gave 0–11 bands for each individual. Every individual had some different bands from the others. Population F had 113 polymorphic bands, Z 93, and B 107. The genetic diversity between Z and B was relatively small whereas there was obvious diversity among F, Z, and B. The band of 600 bp for S01, the band of 278 bp for S02, and the band of 1,400 bp for S14 were specific fragments of population F. S12-900 bp and S12-1400 bp bands were hardly found in Z and B populations, but both were present in F. (Fig. 2 and Fig. 3). It is clear that S1-600 and S14-1400 are polymorphic bands of population F and S6-800 bp is the specific band of Z and B population. S13-750 bp of B is much clearer than that of Z and S5-1500 bp only emerges in B population (Fig. 4).

The percentage of polymorphic loci and the average genetic heterozygosity of every population were calculated according to the statistics of the amplified bands (Table 2).

Genetic Distance & Dendrogram

According to the Nei's expressions, we obtained the genetic distances and the genetic identities among the 3 populations (Table 3). The genetic distances between F and the other two species (0.2998 and 0.02880) are greater than that between Z and B (0.0411). Simultaneously, the genetic identities between Z and B

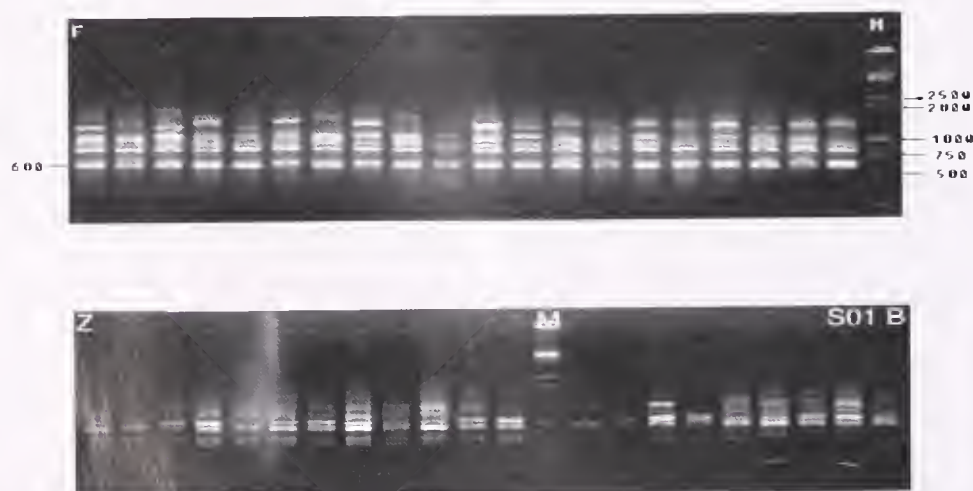


Figure 2. RAPD products of F, Z, B with primer S01 and the 600 bp specific band of F population. M: DNA marker DL-15000 & DL-2000.

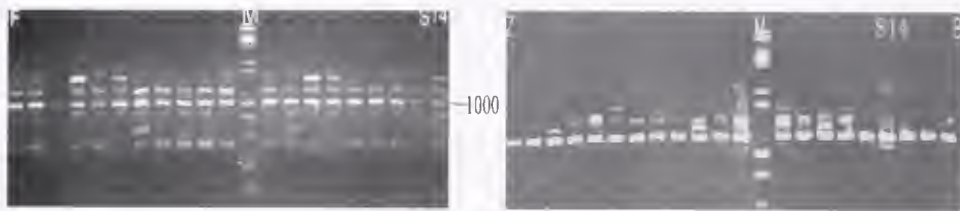


Figure 3. Products of F, Z, B population with primer S14 and 1000 bp polymorphic band of F population. M: DNA marker DL-15000 & DL-2000.



Figure 4. RAPD products of Z, B with primer S05 and S13. M: DNA marker DL-15000 & DL-2000.

TABLE 2.

Percentage of polymorphic loci of three populations and mean heterozygosity.

Population Name	Sample Size	No. of Polymorphic Loci	Mean H	Percentage of Polymorphic Loci (%)
F	20	113	0.1683	53.05
Z	12	93	0.1557	43.66
B	9	107	0.1860	50.23

TABLE 3.

The genetic distances and the genetic identities among populations.

Population Name	F	Z	B
F	***	0.7484	0.7498
Z	0.2898	***	0.9597
B	0.2880	0.0411	***

Genetic identity (above diagonal, Genetic distance (below diagonal))

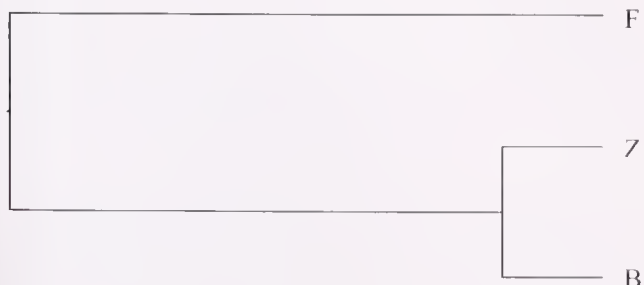


Figure 5. The phylogenetic relationships among the three species of abalone.

was higher than that between F and Z or B. Based on the genetic distances, a phylogenetic tree was constructed with the UPGMA method (Fig. 5). It showed that Z had much closer relationship with B than with F.

DISCUSSION

The average genetic heterozygosities of populations F, Z, and B were 0.1683, 0.1557, 0.1860 respectively, and the percentages of polymorphic loci were 53.05%, 43.66%, and 48.98%. The average genetic heterozygosity was the average number of all amplified loci, and Nei and Li (1979) suggested that genetic heterozygosity was more appropriate for genetic variation. Among the three populations, Z had a relatively lower genetic diversity than B and F. One explanation for this might be that the samples of Zhangzi Island were cultured and therefore had a relatively high homozygosity. Initially the analyses of genetic diversities on marine mollusca were mostly done by means of enzyme technique (Li et al. 2002, Yang et al. 2000). Later, RAPD analysis became a dominant method due to the fact that the RAPD marker is more sensitive than enzyme analysis. Song et al. (1999) analyzed the genetic diversities of *Penaeus japonicus* and found that the percentage of its polymorphic loci was 54.14% and 0.2157 for the average genetic heterozygosity. Wang et al. (2001) concluded that the percentage of polymorphic loci of *Pseudosciaena crocea* was 18.90% and the average genetic heterozygosity was 0.096. The average genetic heterozygosity of *Penaeus chinensis* is 0.2176 (Shi et al. 1999). Compared with the results with other marine creatures, abalone has an accessible genetic variation. Genetic diversity lays the foundation for creatures to survive and adapt to changing environments. Dropping of genetic variation results in lost of the ability of creatures to adjust to the changing living conditions. Protecting biologic diversity is mainly protecting genetic diversity.

The dendrogram constructed on the basis of the genetic distance indicates the systematic relationships among the 3 populations of abalone. Z and B have a very close relationship and their distance is only 0.0411. The distances between F and Z or B are 0.2898 and 0.2880 respectively. According to the traditional classification, F and Z are 2 species of abalone, and they have large differences in many aspects such as morphology, growth environment and growth rate. Z and B are regarded as the same species,

but different geographical subspecies, and they have similar morphology and living habits. Hence, the result of present RAPD analysis is in agreement with the traditional taxonomy, and it is also identical with Thorp's (1982) viewpoint: two populations whose genetic distance is bigger than 0.15 are different species, and the genetic identity between the same species ranges from 0.2 to 0.8.

The RAPD technique has been gradually used in detection of genetic variability, identification of systematic relationship, etc., due to its advantages: prior knowledge of the molecular biology

(genetic background) of the investigated organisms is not required; the required primers are easy to obtain. Our study again suggests that RAPD is an efficient and sensitive genetic marker. However, RAPD also has its defects: poor repetition and artificial identification of the amplified bands. To get reliable results, it is very important to pay attention to the strictness and consistency of experiment conditions, including DNA extraction, concentration test, PCR reaction system establishment etc. If possible the manipulations for all samples to be compared should be done at the same time.

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LACK OF GENETIC DIVERGENCE IN NUCLEAR AND MITOCHONDRIAL DNA BETWEEN SUBSPECIES OF TWO *HALIOTIS* SPECIES

ZHI YONG WANG,^{1,2} KA CHAI HO,¹ DA HUI YU,¹ CAI HUAN KE,³ WAI YAN MAK,⁴ AND KA HOU CHU^{1,*}

¹Department of Biology, The Chinese University of Hong Kong, Hong Kong, China; ²Fisheries College, Jinmei University, Xiamen, Fujian, 361021, China; ³Department of Oceanography, Xiamen University, Xiamen, Fujian, 361005, China; ⁴Molecular Biotechnology Program, The Chinese University of Hong Kong, Hong Kong, China

ABSTRACT *Haliotis discus* and *Haliotis diversicolor* are economically important abalone species in northern and southern China, respectively. Two subspecies have been recognized for each species, namely, *H. discus discus* and *H. discus hannai*, and *H. diversicolor diversicolor* and *H. diversicolor supertexta*. The aim of this study is to elucidate the genetic differences between the two species, and their subspecies, based on sequence analyses of nuclear DNA (first internal transcribed spacer of ribosomal RNA (*ITS-1*) and *18S rRNA*) and mitochondrial DNA (*16S rRNA* and cytochrome oxidase I (*COI*) gene). The two species are identical in their *18S rRNA* sequences but differ in their *ITS-1*, *16S rRNA*, and *COI* sequences, with sequence divergence ranging from 9.3% to 18.5%. Sequences of *18S rRNA* are identical between the subspecies of *H. discus*, and those of *H. diversicolor*. For *16S rRNA* and *COI*, there are 0% to 1.4% divergence between the two *H. discus* subspecies, and 0.2% to 0.6% divergence between the two *H. diversicolor* subspecies. Phylogenetic analyses show that the individual haplotypes of each subspecies are not separated to distinct clades, indicating high genetic similarity between the subspecies of both *H. discus* and *H. diversicolor*.

KEY WORDS: genetic divergence, abalone, *Haliotis discus*, *Haliotis diversicolor*, subspecies

INTRODUCTION

Haliotis discus Reeve, 1846 and *Haliotis diversicolor* Reeve, 1846 are economically important abalone species in northern and southern China, respectively. Two subspecies have been recognized for each species, namely, *H. discus discus* Reeve, 1846 and *H. discus hannai* Ino, 1952 and *H. diversicolor diversicolor* Reeve, 1846 and *H. diversicolor supertexta* Lischke, 1870 (= *H. diversicolor aquatilis* Reeve, 1846) based on morphologic traits (Chen 1984, Nie 1989, Okutani 2001). There have been limited studies on the genetic differences between the two species and their subspecies. Naganuma et al. (1998) reported the differences in partial *18S rRNA* sequences between the 2 subspecies of *H. discus*. No studies have been reported on the genetic differences between these two subspecies based on mitochondrial DNA. In addition, there are no reports on DNA analysis of the two *H. diversicolor* subspecies. The taxonomic status of the subspecies of *H. discus* and *H. diversicolor* is controversial (Hara & Fijio 1992, Geiger 2000). In particular the genetic differentiation between *H. diversicolor diversicolor* in mainland China and *H. diversicolor supertexta* in Taiwan is unknown.

Nuclear DNA markers such as *18S ribosomal RNA* (*18S rRNA*) and the first internal transcribed spacer of *rRNA* (*ITS-1*) and mitochondrial DNA markers such as *16S ribosomal RNA* (*16S rRNA*) and cytochrome *c* oxidase subunit I gene (*COI*) have been extensively used in resolving genetic relationships of various taxonomic groups in the species level (Avisé 1994, Hillis et al. 1996). This study reports the genetic divergence of the four abalone taxa based on sequence analyses of the above mentioned genes.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Haliotis discus discus were collected from Putian County, Fujian Province, China. The cultured stock was introduced from

Japan. *Haliotis discus hannai* were collected from an abalone farm in Qingdao, Shandong Province. Wild *Haliotis diversicolor diversicolor* were collected from Sanya, Hainan Province. *Haliotis diversicolor supertexta* were collected from an abalone farm in Dongshan County, Fujian Province. The stock was introduced from Taiwan. Total DNA was extracted from pleopod muscle using QIAamp DNA Mini Kit (QIAGEN) according to the tissue protocol.

Polymerase Chain Reaction and DNA Sequencing

Segments of *18S rRNA*, *ITS-1*, *16S rRNA*, and *COI* were amplified from two individuals of each taxon using polymerase chain reaction (PCR). Partial *18S rRNA* was amplified using primers 21-mer and 19-mer (Medline et al. 1988) following the conditions as described by Naganuma et al. (1998). PCR conditions for *ITS-1* amplification using primers sp-1–3 and sp-1–5 followed Chu et al. (2001). *16S rRNA* was amplified using the primer pair 16Sar and 16Shr (Simon et al. 1994). PCR reaction cycling program was as follows: 2 min at 94 °C, 38 cycles of 30 sec at 94 °C, 45 sec at 45 °C, 90 sec at 72 °C, and final extension for 5 min at 72 °C. Two primer pairs *COI*f and *COI*a (Palumbi & Benzie 1991) and LCD1490 and HCO2198 (Folmer et al. 1994), were used for amplifying two regions of *COI* gene. The PCR conditions for amplifying *COI* were the same as those used in *16S rRNA* amplification.

Prior to sequencing, PCR products were purified using the QIAquick PCR purification kit. Double-stranded PCR products were sequenced from both directions using both of the primer pairs and ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit. The products were purified and loaded onto an automatic sequencer (ABI PRISM 3100 Genetic Analyzer) for analysis.

Data Analysis

Sequences of each gene were aligned by eye with the aid of ABI Sequence Editor 1.0.3. The sequence of each gene in an individual was confirmed by reference to data from both strands

*Corresponding author. E-mail: kahouchu@cuhk.edu.hk

except the *18S rRNA*. MEGA2 (Kumar et al. 2001) was used in calculating sequence divergence and constructing neighbor-joining phylogenetic trees with 500 bootstrap replicates.

RESULTS

All sequences determined in this study have been deposited in the GenBank database (accession numbers: AY319420-319447). The size of PCR products from *18S rRNA* amplification was approximately 1800 bp, with 620 bp determined from the 5' end and 698 bp determined from the 3' end. The sequences determined in the four abalone taxa were identical.

For *ITS-1*, *16S rRNA*, and *COI* the sequence of each gene was confirmed with reference to data from both strands. The size of PCR product from *ITS-1* amplification was approximately 350 bp. While 340 bp of *ITS-1* were determined from the two subspecies of *H. diversicolor*, 307 bp were determined from the two subspecies of *H. discus*. The sequences from the two individuals in each taxon were identical (Table 1). There was 0.6% (2 bp) sequence divergence between *H. discus discus* and *H. discus hannai*, whereas the sequences from *H. diversicolor diversicolor* and *H. diversicolor supertexta* were identical. The sequence divergence of *ITS-1* between *H. discus* and *H. diversicolor* was 15.9%.

The size of PCR product from *16S rRNA* amplification was approximately 560 bp. While 526 bp of *16S rRNA* were determined from the two subspecies of *H. discus*, 531 bp were determined from the two subspecies *H. diversicolor*. There was 9.4% to 9.6% sequence divergence between *H. discus* and *H. diversicolor*. The sequences of *16S rRNA* between the two *H. discus discus* individuals were identical, whereas for *H. discus hannai*, the sequence of one of the individuals was identical to that of *H. discus discus*, and exhibited 0.4% (2 bp) divergence from the other sequence. There was 0.4% (2 bp) sequence divergence between the two individuals of *H. diversicolor diversicolor*, and also between individuals of *H. diversicolor supertexta*. Between *H. diversicolor diversicolor* and *H. diversicolor supertexta*, the sequence divergence was 0.2% to 0.6% (1–3 bp).

The size of PCR products from *COI* was approximately 680 bp using the primers COII/COIa and 700 bp using LCD1490/HCO2198. In the four taxa, 639 bp of *COI* were determined from the COII/COIa products and 709 bp were determined from the LCD1490/HCO2198 products. The two overlapping segments were combined to a length of 1,272 bp for analysis. There was 17.5% to 18.5% sequence divergence between the two species. Between the two *H. discus discus* individuals, there was 0.6% (7 bp) sequence divergence whereas there was 0.5% (6 bp) sequence divergence between the two *H. discus hannai* individuals. There

was 0.6% to 1.4% (7–18 bp) sequence divergence between two subspecies *H. discus*. The sequence divergence between the two subspecies (*H. discus discus* and *H. discus hannai*) was smaller than that of the sequence divergence between each individual of *H. discus discus*. The sequence divergence was 0.6% (8 bp) between the two *H. diversicolor diversicolor* individuals, and 0.6% (7 bp) between the two *H. diversicolor supertexta* individuals. There was 0.3% to 0.6% (4–8 bp) sequence divergence between the two subspecies of *H. diversicolor*.

Figure 1 shows the neighbor-joining (NJ) phylogenetic trees of the four abalone taxa based on *ITS-1*, *16S rRNA*, and *COI* analyses. Trees constructed based on each of the three genes clearly separated the two species (Fig. 1A to C). In the tree based on *ITS-1* sequence (Fig. 1A), the two subspecies of *H. discus* was clustered into two different clades with over 60 bootstrap support but the two *H. diversicolor* subspecies did not group separately. In the trees based on *16S rRNA* and *COI* (Fig. 1B and C), each of the four taxa did not cluster together, except in the *COI* tree the two *H. discus discus* individuals clustered together. To enhance the phylogenetic signals, the data sets of the three genes were combined for analysis (Fig. 1D). In this tree, the two *H. discus discus* individuals clustered into a single clade with 97 bootstrap support, whereas individuals of the other taxa did not cluster together.

DISCUSSION

18S rRNA is one of the nuclear DNA markers often used for investigating the phylogenetic relationship and evolutionary history of various species. In this study, the partial *18S rRNA* sequences (1308 bp) determined in *H. discus* and *H. diversicolor* are identical. The 3' sequence of the *18S rRNA* segment obtained is identical to that of *H. discus hannai* (390 bp compared, Naganuma et al. 1998; GenBank Accession No. D88571). However, the sequence exhibits 0.5% (2 bp) divergence when compared with the sequence of *H. diversicolor diversicolor* (390 bp compared; D88572) reported by Naganuma et al. (1998). It is unclear why the sequences of *H. discus discus* are different between the two studies. Yet it should be pointed out that not only the *18S rRNA* sequence determined in the present study is identical in the four taxa studied, but it is also identical to that of *H. tuberculata* Linnaeus, 1758 (390 bp compared, AF120511) and exhibits 99.0% homology with the sequence from *H. madaka* (Habe, 1977) (390 bp compared, D88573). Moreover, the 5' sequence of the *18S rRNA* segment obtained from the present study shows 99.8% and 99.3% homology to those of *H. tuberculata* (466 bp compared, AF120511) and *H. rufescens* Swainson, 1822 (445 bp compared, L78885) respectively. The small genetic differences (<1%) be-

TABLE 1.
Percentage divergence of *ITS-1*, *16S rRNA* and *COI* among the four abalone taxa

Taxon	<i>ITS-1</i>				<i>16S rRNA</i>				<i>COI</i>			
	Hdh	Hdd	Hdc	Hds	Hdh	Hdd	Hdc	Hds	Hdh	Hdd	Hdc	Hds
Hdh	0				0.4				0.5			
Hdd	0.6	0			0–0.4	0			0.6–1.4	0.6		
Hdc	15.9	15.9	0		9.4–9.6	9.4	0.4		17.5–18.0	18.2–18.5	0.6	
Hds	15.9	15.9	0	0	9.3–9.4	9.4	0.2–0.6	0.4	17.5–17.8	18.0–18.2	0.3–0.6	0.6

Abbreviations: Hdh: *Haliotis discus hannai*; Hdd: *Haliotis discus discus*; Hdc: *Haliotis diversicolor diversicolor*; Hds: *Haliotis diversicolor supertexta*.

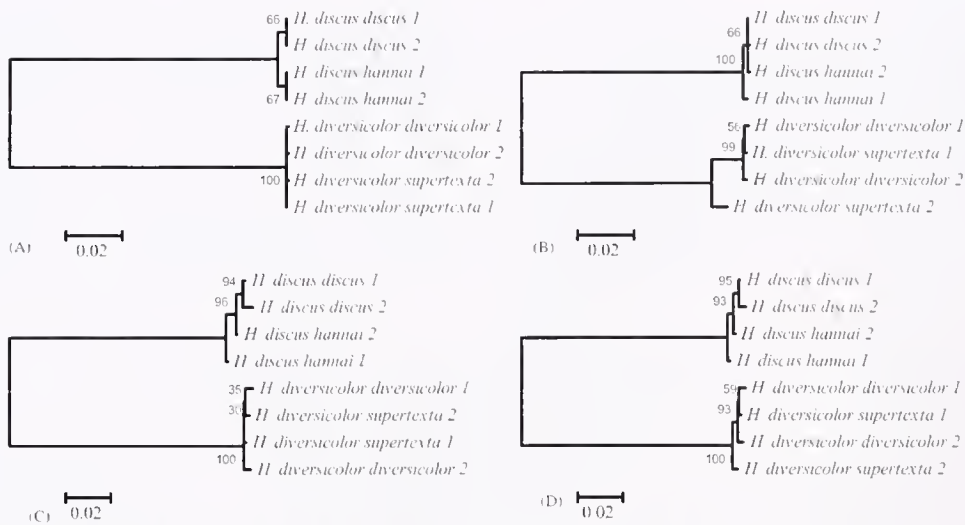


Figure 1. Phylogenetic trees of the 4 abalone taxa resolved by neighbor-joining analyses based on the sequences of A, *ITS-1*; B, *16S rRNA*; C, *COI*; and D, combined data set of three genes.

tween these taxa in *18S rRNA* indicate that this gene is quite conserved among abalone taxa. Therefore, *18S rRNA* is not an appropriate marker in discriminating abalone taxa at the species level, although it may serve as a good marker for phylogenetic studies at higher systematic levels.

Haliotis discus discus and *H. discus hannaï* are recognized to be two subspecies because the ridges on the shell are thin in *H. discus discus* and relatively thicker in *H. discus hannaï*. However, a survey on wild abalone, hatchery-reared abalone seed, and adult abalone from sea ranching (Kobayashi et al. 1992) and growth experiments on the two subspecies under different temperature regimes (Hara 1992), show that the morphologies of the two abalone taxa vary with environmental conditions, particularly temperature. Under low temperature or unstable culture conditions, the morphologic features of *H. discus discus* become similar to those of *H. discus hannaï*. Under high temperature or relatively stable growth conditions, the morphologic features of *H. discus hannaï* become similar to those of *H. discus discus*. Based on the analysis of 12 allozymes, Hara and Fijio (1992) suggested that the two subspecies could not be separated and their morphologic differences are related to the environmental factors in different localities. In the present study, the two subspecies cannot be distinguished from each other based on *18S rRNA*, *16S rRNA*, and *COI* sequence analyses. Their *ITS-1* sequences only differ by 0.6%, indicating low genetic differentiation between these two abalone taxa. In addition, most of the phylogenetic trees do not clearly separate the two subspecies, and thus fail to support the subspecies status of these two abalone taxa.

Similarly, there are only minor morphologic differences between *H. diversicolor diversicolor* and *H. diversicolor supertexta* with the ridges on the shell thicker in *H. diversicolor diversicolor* but thinner in *H. diversicolor supertexta*. Based on our observations, morphologic variations occur among individuals of a population and growth conditions and diet could affect shell morphology of *H. diversicolor* (Wang et al. unpublished data). This is consistent with the observation made by Lü (1978) who concluded that it is difficult to separate the two subspecies. Geiger (2000) indicated *H. diversicolor diversicolor* and *H. diversicolor super-*

texta are synonyms. In the present study, *H. diversicolor diversicolor* and *H. diversicolor supertexta* did not show any distinct differences in their nuclear and mitochondrial DNA sequences. The sequences of *ITS-1*, *16S rRNA* and *COI* of these two subspecies have also recently become available in the GenBank database (Accession Nos. AF296868, AY146396, AY146401, AY146406, AY146397, and AY146402). Comparison with the corresponding sequences obtained from the present study show that the differences are within 0% to 0.34% from each other. The *ITS-1* sequences are identical to those from the present study. There was 0.2% to 0.9% (532 bp compared) and 0.4% to 0.6% (544 bp compared) divergence in *16S rRNA* and *COI*, respectively. Our phylogenetic analyses based on the three genes show that individuals of each of the two subspecies never cluster together. In addition, a parallel study in our laboratories based on AFLP showed there are no diagnostic bands between the two subspecies (Wang et al. 2004, this issue). These results suggest that *H. diversicolor diversicolor* and *H. diversicolor supertexta* are possibly not genetically distinct, thus putting their subspecies status in doubt.

Results from the present study show a lack of genetic divergence between the subspecies of *Haliotis discus* or *Haliotis diversicolor* based on sequences analyses of the four commonly used nuclear/mitochondrial DNA markers. Analysis of other more variable markers such as mitochondrial DNA control region (Chu et al. 2003) should be performed to investigate the genetic differentiation of the four abalone taxa over their geographical range (see, for example, Sites & Crandall 1997) to elucidate their evolutionary relationship and taxonomic status.

ACKNOWLEDGMENTS

The authors thank Dr. Fuhua Li (Institute of Oceanology, Chinese Academy of Sciences) for collecting specimens of *Haliotis discus hannaï* used in this study. The work described in this article was fully supported by grants from Hi-Tech Research and Development (863) Program of China (Project nos. 2001AA620108 & 2003AA603240), and an AoE Fund from The Chinese University of Hong Kong.

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GENETIC VARIATIONS AND DIVERGENCE OF TWO *HALIOTIS* SPECIES AS REVEALED BY AFLP ANALYSIS

ZHI YONG WANG,^{1,2*} CAI-HUAN KE,³ YI-LEI WANG,¹ ZHI-QUN XIAO,¹ KA CHAI HO,²
AND KA HOU CHU²

¹Fisheries College, Jimei University, Xiamen, Fujian, 361021, China; ²Department of Biology, The Chinese University of Hong Kong, Hong Kong, China; ³Department of Oceanography, Xiamen University, Xiamen, 361005, China

ABSTRACT Amplified fragment length polymorphism (AFLP) technology was used to reveal genetic variations and divergence of *Haliotis discus* and *H. diversicolor*, the two economically important abalone species in China. Three sets of selective primers were used for amplification, and 350 bands ranging in size from 80 bp to about 500 bp were scored. The mean percentages of polymorphic bands in the four taxa varied from 51.7% to 62.7%. Similarity indices and genetic distances between individuals of the same species were greater than 0.8 and smaller than 0.2, respectively, whereas the values among individuals of different species were smaller than 0.3 and greater than 1.2, respectively. AFLP profiles of the two abalone species were distinct and could be used for species identification. Specific AFLP bands were also identified for discriminating the two subspecies of *H. discus*. Yet no diagnostic bands were found to differentiate the two subspecies of *H. diversicolor*. The lack of genetic divergence between these two subspecies supports results from a parallel study based on nuclear and mitochondrial DNA sequence analyses. We suggest that the subspecies status of *H. diversicolor diversicolor* and *H. diversicolor supertexta* is questionable. Results of this study are applicable to resource management and future genetic improvement of the two abalone species in China.

KEY WORDS: AFLP, abalone, genetic variation, genetic divergence, *Haliotis discus*, *Haliotis diversicolor*, subspecies

INTRODUCTION

Haliotis discus Reeve, 1846 and *Haliotis diversicolor* Reeve, 1846 (Mollusca: Gastropoda: Orthogastropoda: Vetigastropoda: Haliotoidea: Haliotidae; *Haliotis*) are two economically important abalone species that are extensively cultured in China and Japan (Chen 1984). Based on morphologic characters, two subspecies have been identified from each species (Chen 1984, Nie 1989, Okutani 2001). They are *H. discus discus* Reeve, 1846 and *H. discus hannai* Ino, 1952, and *H. diversicolor diversicolor* Reeve, 1846 and *H. diversicolor supertexta* Lischke, 1871 (also named *H. diversicolor aquatilis* Reeve, 1846). However, analyses based on sequences of mitochondrial and nuclear DNA markers show that the subspecies of each species are not genetically distinct, although the difference between the two species is evident (Wang et al. 2004b, this issue). Because the taxonomic status of the subspecies of *H. discus* and *H. diversicolor* is controversial, we aim to further elucidate any genetic differences between these taxa using molecular markers with a higher resolving power.

AFLP is a powerful technique applicable for genomic DNA fingerprinting (Vos et al. 1995). It has a higher resolving power and sensitivity than RAPD analysis in revealing allelic polymorphism (Barker et al. 1999) and thus has been suggested to be one of the appropriate techniques for revealing the genetic variation within species. This technique has been widely applied in studies of genotyping, population differentiation, and genetic diversity for a wide variety of organisms (Liu et al. 1998, Lerceteau & Szmidt 1999, Mueller & Wolfenbarger 1999, Seki et al. 1999, Keiper & McConchie 2000, Wang et al. 2000). In the present study, the genetic variation and divergence within and between the two abalone species *H. discus* and *H. diversicolor* were investigated using AFLP technique. For breeding purpose and genetic resource management, it is essential to clarify the genetic variation and relationship of these two commercially important taxa.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Specimens of *H. discus discus*, *H. discus hannai*, *H. diversicolor supertexta*, were collected from abalone farms in Putian, Fujian Province (introduced from Japan), Qingdao, Shandong Province, and Dongshan, Fujian Province (introduced from Taiwan), China, respectively. Specimens of *H. diversicolor diversicolor* were collected from the wild stock in Sanya, Hainan Province. The specimens were either preserved in 95% ethanol or stored in -80°C prior to analysis. Total DNA was extracted from foot muscle (20–25 mg) using proteinase-K digestion and DNA binding columns (QIAGEN QIAamp DNA Mini Kit) according to the manufacturer's instructions. The quality of extracted DNA was assessed using 1.0% agarose gel electrophoresis, and the DNA concentration was measured with an UV spectrophotometer (Hitachi U-2001).

AFLP Reactions

Procedures and reagents for AFLP analysis were applied as described in Vos et al. (1995). AFLP banding patterns were visualized on 5% denaturing polyacrylamide gel using silver staining technique. DNA templates for AFLP reactions were generated by restriction digestion and ligation. About 100 ng of total DNA was digested with 5U of *EcoRI* and *MseI* (New England BIOLABS) in 1X NE buffer2 at 37°C for 2 h. The digested DNA fragments were ligated with 2.5 pmol of *EcoRI* and 25 pmol *MseI* adapters in a reaction mixture containing 0.25 mg BSA, 5 pmol ATP, 0.04U T4Dnase, and 10X NE buffer2 at 37°C for 6 h. The ligated DNA fragments were preamplified using a MJ Thermocycler (PTC-100) with a pair of primers containing a single selective nucleotide. The preamplification PCR reaction was conducted at an annealing temperature of 53°C for 30 sec. The 20- μL PCR product mixture was diluted 10-fold with distilled water and used as templates for the subsequent selective PCR amplification. The selective amplifica-

*Corresponding author. E-mail: zywang@jmu.edu.cn

TABLE 1.

Numbers of amplified bands scored and polymorphic bands observed from AFLP analysis of 4 taxa of *Haliotis* using 3 sets of selective primers.

Taxa	No. of Specimens	Total No. of Bands	Polymorphic Bands		No. of Genotypes
			No.	%	
<i>H. discus discus</i>	10	231	145	62.7	10
<i>H. discus hannai</i>	8	205	106	51.7	8
<i>H. diversicolor diversicolor</i>	10	195	107	55.0	10
<i>H. diversicolor supertexta</i>	10	196	112	57.1	10

tion was performed using 3 pairs of primers (*E*-AAG/*M*-CGA, *E*-AGG/*M*-CTG and *E*-AGC/*M*-CTT).

Gel Electrophoresis and Silver Staining

After selective amplification, the PCR products were mixed with 10 μ L AFLP loading buffer (99% formamide, 10 mM EDTA, 0.05% bromophenol and 0.05% xylene cyanol). The product mixtures were denatured and concentrated at 90°C for 25 min, and cooled down in an ice bath immediately after denaturation. A 5% denaturing polyacrylamide gel (4.75% acrylamide, 0.25% bis acrylamide, 7 mol \times 1⁻¹ urea and 0.5X TBE) was prerun at 145 W for 30 min. Each well was loaded with 2.5 μ L of sample. The gel was electrophoresed in a BioRad Sequi-Gen GT DNA sequencing cell (38 \times 50 cm) at 110 W and 50°C for 2.5 h.

Bands on the gel were visualized using silver staining method derived from Merrill et al. (1979). After electrophoresis, the gel was fixed in 1% ethanoic acid for 30 min. The fixed gel was rinsed in distilled water and stained with a mixture of 0.2% silver nitrate and 0.007% benzene sulphonic acid for 30 min. The stained gel was rinsed again and immersed in a developing solution (2.5% sodium carbonate, 0.037% formaldehyde, 0.002% sodium thiosulphate). When the bands became clear, the development was stopped with 1% ethanoic acid. The bands were scored using the Gel Imaging Analyzing System (Kodak Digital Science, EDAS-120) and the band sizes were estimated with a standard AFLP DNA ladder (Invitrogen, Life Technologies).

Data Analysis

AFLP bands were scored for presence (1) or absence (0), and transformed into 0/1 binary character matrix. Based on the binary matrix, genotypes, percentage of polymorphic bands, similarity indices, and genetic distance were obtained. Similarity indices were calculated using the formula $S = 2N_{ab}/(N_a + N_b)$ (Nei & Li 1979), where N_a and N_b are the number of bands in individuals a and b, respectively and N_{ab} is the number of shared bands. Genetic distances were computed using the formula $D = -\ln S$ (Nei & Li 1979). For the calculation of the above values, we have developed a Microsoft Excel-based BASIC program (AFLP Data Analyser v1.3). Cluster analysis based on UPGMA was conducted using the software MEGA 2 (Kumar et al. 2001) based on the distance matrix.

RESULTS

AFLP was performed using three pairs of selective primers on a total of 38 individuals belonged to four abalone taxa. A total of 350 different bands ranged from 80 to 500 bp in size were scored. The number of bands generated from a single individual varied

from 116 to 163. Because no two individuals shared exactly the same banding pattern, the number of genotypes identified was the same to the number of individuals used in the study (Table 1). The number of bands generated from each taxon varied from 195 to 231 and the percentage of polymorphism ranged from 51.7% (in *H. discus hannai*) to 62.7% (in *H. discus discus*).

A representative AFLP fingerprint from the amplification using one of the primer sets (Fig. 1) shows that the fingerprint was quite similar among individuals of the same species but distinct between different species. Among the 350 bands scored, only 10 bands (2.9%) were shared among all individuals of the four taxa. There were 79 bands common to all individuals of *H. discus*, constituting 32% of the total number of bands scored in this species. On the other hand, 77 bands were common to all *H. diversicolor*, accounting for 36% of the total number of bands scored in this species. There were 41 bands specific to all individuals of *H. discus* but not found in *H. diversicolor*, and 34 bands were specific to *H. diver-*

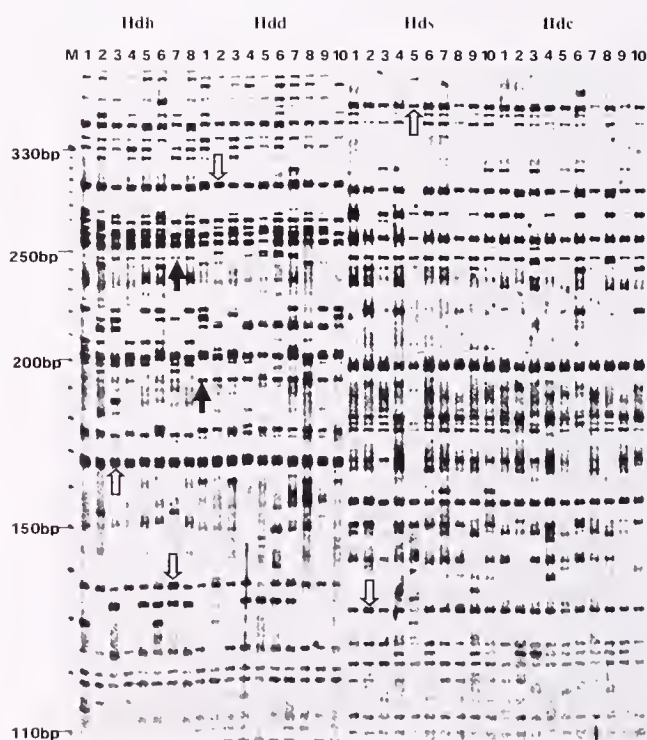


Figure 1. AFLP patterns using the primer set *E*-AAG and *M*-CGA. Hdh: *H. discus hannai*; Hdd: *H. discus discus*; Hds: *H. diversicolor supertexta*; Hdc: *H. diversicolor diversicolor*; M: AFLP DNA ladder. White arrows indicate bands specific to a species; black arrows indicate bands specific to a subspecies.

TABLE 2.

Comparison of similarity index (above diagonal) and genetic distance (below diagonal, in parentheses) within and between taxa estimated by AFLP banding pattern in the four taxa of *Haliotis* abalone.

	<i>H. discus discus</i>	<i>H. discus hannai</i>	<i>H. diversicolor diversicolor</i>	<i>H. diversicolor supertexta</i>
<i>H. discus discus</i>	0.8295 (0.1870)	0.7756	0.2645	0.2600
<i>H. discus hannai</i>	(0.2542)	0.8484 (0.1645)	0.2510	0.2404
<i>H. diversicolor diversicolor</i>	(1.3299)	(1.3825)	0.8484 (0.1644)	0.8348
<i>H. diversicolor supertexta</i>	(1.3470)	(1.4255)	(0.1841)	0.8401 (0.1743)

sicolor. Some of these bands are indicated in Figure 1. These species-specific bands could be used for the development of molecular markers applicable in the identification of these two species of abalone. Six diagnostic bands were also identified between *H. discus discus* and *H. discus hannai*, with three specific to each of the taxa. Yet for *H. diversicolor*, no bands specific to each of the subspecies could be identified.

The similarity indices and genetic distances within or between the four taxa of abalone based on AFLP banding patterns are shown in Table 2. The similarity indices between individuals of the two abalone species were all lower than 0.3, with genetic distances greater than 1.3. The mean similarity index between individuals of *H. discus discus* and *H. discus hannai* (0.78) was considerably lower than those among individuals of the same subspecies. It is evident that the two subspecies of *H. discus* are genetically distinct. However, the similarity index and genetic distance between individuals of *H. diversicolor diversicolor* and *H. diversicolor supertexta* were very similar to values among individuals of the same subspecies, suggesting a lack of genetic divergence between these two taxa.

A phylogenetic tree of the 38 individuals of the four abalone taxa was constructed using UPGMA method (Fig. 2). Individuals of *H. discus discus* and *H. discus hannai* were separated into two distinct clusters but individuals of *H. diversicolor diversicolor* or *H. diversicolor supertexta* did not cluster together.

DISCUSSION

AFLP technique is a highly effective method for examining genetic polymorphism. It does not require any prior sequence information of the species under study and a large number of loci can be generated from a small amount of DNA. Due to its high level of polymorphism, the technique is appropriate to assess organisms with low level of genetic divergence. It has extensively been applied in genetic studies in diverse groups of organisms (Liu et al. 1998, Lerceteau & Szmidt 1999, Mueller & Wolfenbarger 1999, Seki et al. 1999, Keiper & McConchie 2000, Wang et al. 2000, Nicholas et al. 2001, Ogden & Thorpe 2002). In the present study, we identified 195 to 231 different bands from a single abalone taxon by using three primer sets in the four taxa. Each primer set generates 65 to 77 bands from a single taxon, with polymorphism higher than 50%. Further, AFLP patterns of the individuals differed from each other, and the pattern from a single primer set could be used to distinguish all the individuals from the same taxon. Previous studies, based on analyses of allozymes and RFLP of mtDNA, suggest low genetic diversity in abalone populations,

particularly the cultured stocks (Fujino 1978, Smith & Conroy 1992). No genetic variations were found in *H. discus hannai*, *H. discus discus*, and *H. diversicolor supertexta* in a recent study in our laboratory using 12 allozyme loci and 15 individuals of each taxon (Ke et al. 2003). Yet, more than half of the AFLP bands scored in the present study were found to be polymorphic (the same specimens of *H. discus discus* and *H. diversicolor supertexta* were used in both studies), indicating AFLP as a more effective approach than allozyme analysis in detecting genetic diversity in abalone. Moreover, the results suggest that there are no differences in genetic diversity between the wild stock of *H. diversicolor*

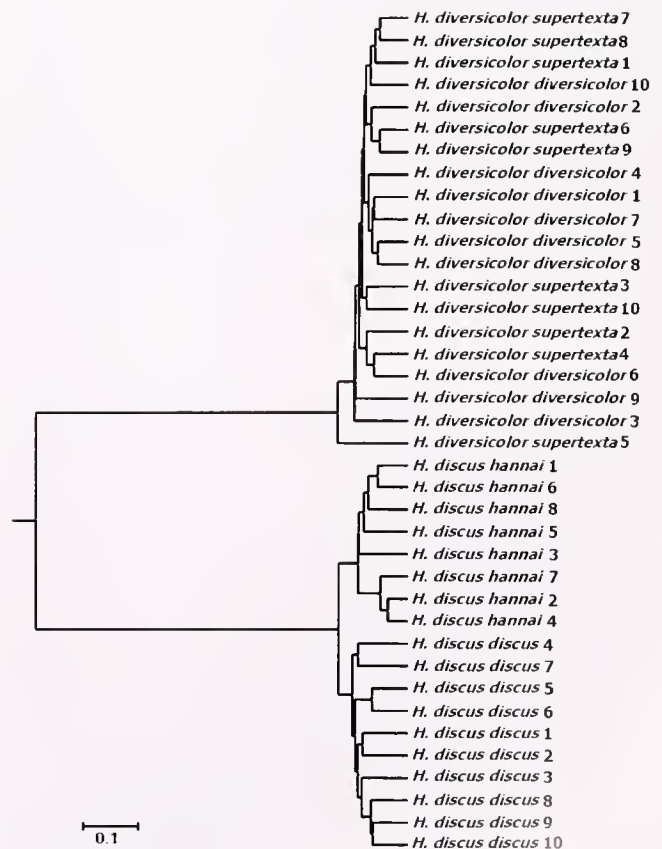


Figure 2. UPGMA dendrogram of the four taxa of *Haliotis* abalone based on distance matrix from AFLP analysis (Table 2).

diversicolor and the cultured stocks of the other three taxa. It is difficult to distinguish whether the wild stock of *H. diversicolor* exhibits low genetic diversity due to over-fishing, or the cultured stocks of the other taxa do not suffer from a reduction of genetic diversity. Further studies on genetic differentiation among abalone populations are needed to understand the extent of genetic polymorphism in wild stocks, as well as effect of aquaculture on genetic diversity of abalone. Nevertheless, the present study establishes AFLP as the appropriate method in elucidating and monitoring genetic polymorphism in abalone.

AFLP can be applicable in discriminating different species or subspecies but also elucidating their evolutionary relationships (Tsoi et al. 2003, Wang et al. 2004a). The present study shows that the difference in AFLP banding pattern between *H. discus* and *H. diversicolor* is apparent, giving a genetic distance of higher than 1.3 between the two species. Our phylogenetic analysis also shows that the individuals of these two taxa are clearly separated into two clusters. The results are consistent with the analyses based on morphologic traits or mitochondrial DNA (Wang et al., 2004b, this issue), thus indicating AFLP technique as a potentially useful method for species identification and phylogenetic analysis among species. We have isolated some of the AFLP bands specific to a particular abalone taxon for sequencing analysis and the development of sequence tagged sites (STS) markers. These markers will be most useful for the genetic identification and breeding studies of the abalone taxa under study.

Haliotis discus discus and *H. discus hannai*, and *H. diversicolor diversicolor* and *H. diversicolor supertexta* have been designated as subspecies of their respective species based on morphologic traits (Chen 1984, Nie 1989, Okutani 2001). Results from the present study show that AFLP banding patterns of *H. diversicolor diversicolor* and *H. diversicolor supertexta* are similar. No bands specific to each taxon could be identified. The genetic distance between these two taxa is similar to that within each taxon. Phylogenetic analysis shows that individuals within each subspecies do not cluster together, suggesting *H. diversicolor diversicolor* and *H. diversicolor supertexta* are genetically similar. Recent studies in our laboratory on these two taxa using eight additional primer sets also support results from the present study using three primer sets. A parallel study on the sequence analysis of nuclear and

mitochondrial (mt) genes (*18S rRNA*, *ITS-1*, *mt 16S rRNA*, and cytochrome oxidase I) also shows a lack of divergence between *H. diversicolor diversicolor* and *H. diversicolor supertexta* (Wang et al. 2004b, this issue). Investigations based on morphologic traits and allozymes also cannot establish differences between these two taxa (Lü 1978, Ke et al. 2003 and unpublished data).

In contrast to our AFLP data on *H. diversicolor*, AFLP analysis on the two subspecies of *H. discus* show that they are genetically distinct. Diagnostic bands could be identified from both *Haliotis discus discus* and *H. discus hannai*, and genetic distance among subspecies is higher than values within subspecies. Phylogenetic analysis also demonstrates that individuals of these two subspecies are separated into two different clusters. Yet it should be noted that nuclear and mtDNA sequence analyses failed to distinguish these two subspecies (Wang et al. 2004b, this issue), showing that AFLP is a more sensitive assay for discriminating genetic divergence in closely related taxa. Taken together, results from the present study suggest that because the subspecies status of *Haliotis discus discus* and *H. discus hannai* may be warranted, the distinction between *H. diversicolor diversicolor* and *H. diversicolor supertexta* is put into question. The latter conclusion supports Geiger's (2000) assertion that the two subspecies of *H. diversicolor* are synonymous. Yet Hara and Fijio (1992) also argued that divergence of the two subspecies of *H. discus* only represents population differences. It should be pointed out that only a single population from each of the four abalone taxa was included in the present study. A more extensive study based on population aggregation analysis (Davis & Nixon 1992) over the geographical range of these abalone taxa is necessary to establish the presence or absence of genetic divergence of the subspecies in question.

ACKNOWLEDGMENTS

The authors thank Dr. Fuhua Li (Institute of Oceanology, Chinese Academy of Sciences) for collecting the specimens of *Haliotis discus hannai* used in this study. The work described in this article was fully supported by grants from Hi-Tech Research and Development (863) Program of China (Project nos. 2001AA620108 & 2003AA603240, and an AoE Fund from The Chinese University of Hong Kong).

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GENETIC CONTROL OF BLuish SHELL COLOR VARIATION IN THE PACIFIC ABALONE, *HALIOTIS DISCUS HANNAI*

TOSHIMASA KOBAYASHI,¹ IKUE KAWAHARA, OSAMU HASEKURA² AND
AKIHIRO KIJIMA³

¹Iwate Fisheries Technology Center Heita Kamaishi Iwate 026-0001 Japan; ²Iwate Inland Fisheries Technology Center Yoriki Matuo Iwate 028-7302 Japan; ³Laboratory of Integrate Aquatic Biology, Marine Field in Field Science Center, Graduate School of Agricultural Science, Tohoku University Onagawa Miyagi 986-2242 Japan

ABSTRACT Shell color variation is one of the more interesting phenomena in abalone breeding. Especially, the identification of inheritable shell color will be a useful visual genetic marker. Bluish shell colored individuals were discovered in one full-sib family A out of nine families (A to I) in the Pacific abalone, *Haliotis discus hannai*. The segregation of the variant (bluish) type and the normal (greenish) type was 13:27, suggesting the existence of a recessive allele at a single locus. To clarify the genetic control of shell color variation, mating experiments were designed for the F₂ generation among the bluish-type and greenish-type individuals within the full-sib family A. All individuals were of the greenish type in the three full-sib families between greenish-type female and greenish-type male, whereas all individuals were of the bluish type in the six full-sib families between bluish-type female and bluish-type male. All individuals were of the greenish type in the four full-sib families between bluish-type female and greenish-type male, whereas segregation of the greenish type and the bluish type was observed in the two full-sib families between greenish-type females and bluish-type males. The observed numbers showed no significant deviation from expected numbers calculated under Mendelian segregation (1:1) in each family. These results indicate that the bluish and greenish shell color variant types are controlled by a recessive allele (*b*) and a dominant allele (*G*) at a single locus. Therefore, the genotype of parental individuals (f1 and m1) of the full-sib family A is estimated as *G/b*. To elucidate the genetic control of shell color variation, further mating experiments were designed for the F₂ generation within and between full-sib families D and G in which the male parent (m1) is common with the family A. As expected, segregation of the bluish and greenish types was observed in the two full-sib families out of 13 families. The observed numbers showed no significant deviation from expected numbers calculated under Mendelian segregation (3:1). These results support the elucidation of the genetic control of shell color variations described in the present study.

KEY WORDS: abalone, *Haliotis discus hannai*, shell color variation, mating experiments

INTRODUCTION

Shell color variation is one of the most interesting phenomena in shellfish aquaculture. The genetic basis of shell color variation was reported in *Urosalpinx cinerea* (Cole 1975), *Mytilus edulis* (Newkirk 1980), *Thais emarginata* (Palmer 1985), *Pinctada fucata martensii* (Wada & Komaru 1990), *Fulvia mutica* (Fujiwara 1995), and *Ruditapes philippinarum* (Kisbioka et al. 1997). Cole (1975) reported that in mating experiments a single-locus genetic model controlled three types of shell color variation in *Urosalpinx cinerea* with complete dominance. Similarly, Newkirk (1980) reported that at least two types of shell color variants occurs in *Mytilus edulis* and is determined by a simple genetic mechanism.

The Pacific abalone (*Haliotis discus hannai*) is a commercially valuable species. The creation of artificial culture strains has been expected to allow discrimination from the wild individuals. Visual genetic markers, such as color variation, are, therefore, desired. Shell color variation has been reported in a number of abalone species (Ino 1952, Sakai 1962, Ogino & Ohta 1963, Sibui 1971), however, the variation has been shown to be dependent on dietary differences. In the Pacific abalone, *Haliotis discus hannai*, Sakai (1962) observed bluish-green, green, greenish-brown, and brown shell colors produced by different alga diets. Ogino and Ohta (1963) reported that a green colored shell may be obtained through an artificial diet, however there has been limited research to determine the genetic basis of shell color variation in the Pacific abalone.

In an attempt to underpin the genetic control of traits such as color variation, the discovery of color variation and detection of color types are first needed, followed by segregation of phenotypes

in offspring produced by systematic mating experiments under the same rearing conditions. In the present study, bluish shell color-variant individuals were discovered in one full-sib family (one male crossed with one female). Systematic mating experiments were then undertaken and segregation of the color types were analyzed in the offspring of several mating experiments in an attempt to understand the genetic control of shell color variation in the Pacific abalone.

MATERIALS AND METHODS

Detection of the Color Types

Shell color types were visually divided into two groups, one of which was a greenish type (green to bluish-green but not light and purplish blue) and the other a bluish type (light-blue to purplish-blue with greenish color) as shown in Figure 1. Determination of shell color types of the offspring in experiments I, II, and III were carried out using individuals of more than 17 mm of shell length at the ages of 229, 222, and 245 days after fertilization, respectively. Because, shell color types are clearly divided into two types at more than 17-mm shell length. Any changes of shell color types were not observed after the detection.

Mating Experiments

Figure 2 shows the mating experiment system used in the present study. Two populations were collected from the coastal water of Aomori (41°N, 141°E) and Iwate (40°N, 142°E) Prefectures in the northeastern part of Japan. Aomori population was stocked during two generations, and Iwate population was stocked during three generations before using. One greenish fe-

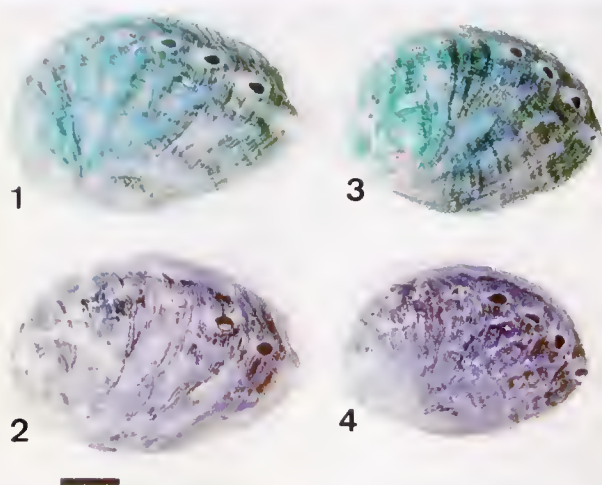


Figure 1. Typical shell color variation of *Haliotis discus hannai* of mating experiment I and II. 1; greenish shell color type of family A of mating experiment I. 2; bluish shell color type of family A of mating experiment I. 3; greenish shell color type of family AA12 of mating experiment II. 4; bluish shell color type of family AA12 of mating experiment II. Individuals of 1 and 2 are approximately two years old, 3 and 4 are approximately 17 months old. Scale bar = 10 mm.

male (Pf) of the Aomori population and one greenish male (Pm) of the Iwate population were used for producing the parental generation (P₁) during 1996. Three greenish females and three greenish males of the P₁ generation were used for producing the nine full-sib families of the first offspring generation (F₁) in September 1999 as shown in mating experiment I.

During mating experiment II, fifteen pair-crosses were produced in August 2001 using the 6 females (3 individuals of the bluish-type and 3 of the greenish-type) and 5 males (3 individuals of the bluish-type and 2 of the greenish-type) from the full-sib family A in which bluish and greenish color types were segregated. Combinations of the crossed individuals were as follows (Fig. 2): bluish-type female × bluish-type male (6 pair), bluish-type female × greenish-type male (4 pair), greenish-type female × bluish-type male (2 pair), and greenish-type female × greenish-type male (3 pair).

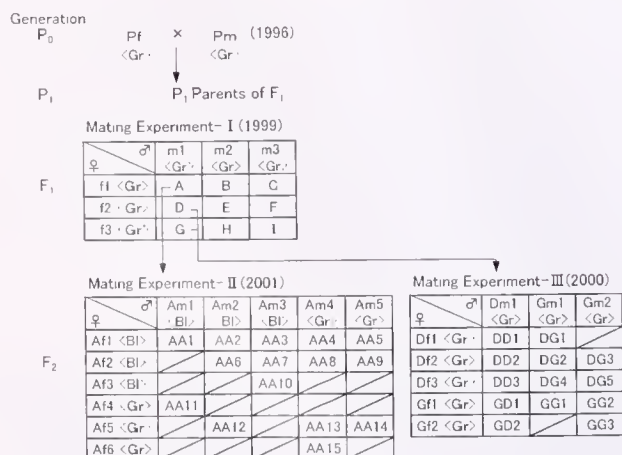


Figure 2. Mating experiment system in the present study. Gr and Bl represent phenotype of greenish-type and bluish-type, respectively.

To elucidate the genetic control of shell color variation, mating experiment III was designed for the F₂ generation within and between full-sib families D and G in which male parents are common with full-sib family A. Thirteen full-sib families were produced in November 2000 using five females and three males as shown in Figure 2.

Breeding and Rearing

Individuals of parents for mating were set into the separate containers before spawning. Spawning was artificially induced with ultraviolet light-irradiated seawater. The spawned eggs were separated into several containers for each pair-mating, and fertilized with the sperm of a different male, respectively. After hatching, larvae were reared in the meshed bottom containers for 4 days. Settlements were conducted with wavy-plates, which were covered with *Ulva* lens, and placed into clear plastic 20-L containers. They were reared in a sunny place allowing juveniles to feed on diatoms, which grew on the wavy-plates and the surfaces of the containers. An artificial diet (Cosmo Kaihatsu K.K., Standard type), which usually produced greenish color shells on the Pacific abalone, was given after their shell lengths reached about 10 mm. They were reared in running seawater whose temperature was maintained at almost 20°C during the experiments.

Statistic Analysis

Expected numbers were calculated under Mendelian segregation, assuming that the bluish type was controlled by a recessive allele at a single locus. The χ^2 test was performed for comparison between the observed number and the expected one.

RESULTS

In the present study, the bluish color shell was formed after the artificial diet provided, although it was observed that diatoms and an artificial diet normally formed a greenish color shell. The bluish-type individuals initially formed green shells until their shell length reached about 10–15 mm, therefore around an apex of their shells remained greenish color (Fig. 1). The ranges of shell length determined the shell color types in the mating experiment I, II, and III were 17–36 mm, 17–46 mm, and 17–37 mm. The shell's bluish pigment appeared only on the outer shell surface, though the hues of the pearl layer were not changed.

The results of the mating experiment I are shown in Table 1. Nine full-sib families were obtained and only the greenish type was observed in all families except one. In full-sib family A, phenotypic segregation was observed; that is, 27 were of the greenish type, whereas 13 were the bluish type. The results showed no significant deviation from expected numbers calculated under Mendelian segregation (3:1).

Three females and three males of bluish type, and three females and two males of greenish type were then picked up from full-sib family A, and a total of 15 full-sib families of the F₂ generation were produced. The results of the mating experiment II are shown in Table 2. All offspring individuals were of the greenish type in the three full-sib families (AA13, AA14, and AA15) produced by mating between greenish-type females and greenish-type males and in the four full-sib families (AA4, AA5, AA8, and AA9) between bluish-type females and greenish-type males. On the other side, all offspring individuals were of the bluish type in the six full-sib families (AA1, AA2, AA3, AA6, AA7, and AA10) produced by mating between bluish-type females and bluish-type

TABLE 1.

Segregation of phenotypes of offspring in each full-sib family of the Mating Experiment I.

♀/♂	Observed (Expected)					
	m ₁ (Gr)		m ₂ (Gr)		m ₃ (Gr)	
	Gr	Bl	Gr	Bl	Gr	Bl
f ₁ (Gr)	A		B		C	
	27	13	43	0	49	0
	(30.0)	(10.0)				
	$\chi^2 = 1.20$					
f ₂ (Gr)	D		E		F	
	107	0	34	0	64	0
	G		H		I	
f ₃ (Gr)	39	0	37	0	95	0

Gr and Bl represent the phenotype of greenish-type and bluish-type, respectively.

males. Phenotypic segregation of the greenish type and bluish type was observed in the two full-sib families (AA11 and AA12) between greenish-type females and bluish-type males. The observed numbers showed no significant deviation from expected numbers calculated under Mendelian segregation (1:1) in each family. These results suggest that the bluish and greenish shell color variant types are controlled by recessive allele (*b*) and dominant allele (*G*) at a single locus, and the genotype of parental individuals in the pair-cross A are estimated as *G/b*.

To elucidate the genetic control of shell color variation, further mating experiments (mating experiment III) were designed for the F2 generation within and between full-sib families D and G in

TABLE 2.

Segregation of phenotypes of offspring in each full-sib family of the Mating Experiment II.

Cross Type	Observed (Expected)		
	Gr	Bl	
Gr × Gr			
AA ₁₃	134	0	
AA ₁₄	64	0	
AA ₁₅	184	0	
Gr × Bl			
AA ₁₁	40 (36.0)	32 (36.0)	$\chi^2 = 0.89$
AA ₁₂	71 (73.5)	76 (73.5)	$\chi^2 = 0.17$
Bl × Gr			
AA ₄	48	0	
AA ₅	163	0	
AA ₈	80	0	
AA ₉	35	0	
Bl × Bl			
AA ₁	0	29	
AA ₂	0	205	
AA ₃	0	69	
AA ₆	0	93	
AA ₇	0	100	
AA ₁₀	0	102	

Gr and Bl represent the phenotype of greenish-type and bluish-type, respectively.

which the male parent (m1) is the same as that of the full-sib family A (Table 3). All individuals in all full-sib families were of the greenish type except the two families. The bluish type appeared in the DG3 and GG3 families. The observed numbers of greenish to bluish individuals each of the DG3 (56:14) and GG3 (85:24) families showed no significant deviation from the expected numbers calculated under Mendelian segregation (3:1).

DISCUSSION

Shell color variations reported in the Pacific abalone are caused by environmental factors, such as natural and/or artificial dietary food. In the mating experiment I of the present study, however, bluish-type individuals were discovered along with the greenish type in one family, even though all individuals in the family have been reared in the same tank (same food and same environment). This suggests that color variations are caused by genetic factors. Moreover, the segregation rate of greenish and bluish types was not significantly different from 3:1, suggesting an existence of dominant (*G*) and recessive (*b*) alleles at a single locus. Also, the genotypes of both parents, f1 and m1, are considered to be *G/b*.

To elucidate this assumption, mating experiments should be conducted between any combinations of color types within family A. Because an abalone spawns a high number of eggs, all combinations could be designed in the same generation and in the same family as in the present study. In mating experiment II, the results of segregation indicated that the assumptions of genotypes are elucidated without any exception. For example, the genotypes of *G/G* and *G/b* would be included in the greenish-type individuals of family A at the proportion of 1:1 in this assumption. As a matter of course, the genotypes of bluish-type parental individuals are estimated as *b/b*. Then, the genotypes of maternal greenish-type individual of family AA11 and AA12 are estimated as *G/b*, because, both families segregated no significant deviation from 1:1.

Similarly, the genotypes of *G/G* and *G/b* would be included in the B, C, D, and G families at the proportion of 1:1. The results of

TABLE 3.

Segregation of phenotypes of offspring in each full-sib family of the Mating Experiment III.

♀/♂	Observed (Expected)					
	Dm ₁ (Gr)		Gm ₁ (Gr)		Gm ₂ (Gr)	
	Gr	Bl	Gr	Bl	Gr	Bl
Df ₁ (Gr)	DD1		DG1			
	190	0	227	0	—	—
Df ₂ (Gr)	DD2		DG2		DG3	
	107	0	29	0	56	14
					(52.5)	(17.5)
					$\chi^2 = 0.93$	
Df ₃ (Gr)	DD3		DG4		DG5	
	188	0	136	0	133	0
Gf ₁ (Gr)	GD1		GG1		GG2	
	113	0	106	0	84	0
Gf ₂ (Gr)	GD2				GG3	
	79	0	—	—	85	24
					(81.7)	(27.3)
					$\chi^2 = 0.52$	

Gr and Bl represent the phenotype of greenish-type and bluish-type, respectively.

mating experiment III show that all segregations in 13 combinations from the D and G families are not contradicted under the Mendelian Law assuming a single locus. From the earlier results, genetic color variations observed in the Pacific abalone population, and these color variations are controlled by dominant (*G*) and recessive (*b*) alleles at a single locus.

The color variation of the bluish type has not been observed or reported in the natural resources of abalone around Japan. It could be caused by a low frequency of the allele (*b*), by a newly arisen mutation of allele (*b*), and/or by the low adaptation (low viability) of an individual of homozygote (*b/b*). The clarification of this issue provides a subject for future investigation.

In the present abalone culture, the development of new artificial strains is expected and can be distinguished at a glance. The bluish type shell color variation will be useful for future abalone culture as a visual genetic marker for several breeding studies.

ACKNOWLEDGMENTS

This study was supported in part by Grant-in-Aid from the Ministry of Agriculture, Forestry and Fisheries (Development of Fundamental Technologies for Effective Genetic Improvement of Aquatic Organism).

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THE IDENTIFICATION OF GENETIC RESISTANCE TO AMYOTROPHIA IN JAPANESE ABALONE, *HALIOTIS DISCUS DISCUS*

MOTOYUKI HARA,^{1,*} MASASHI SEKINO,² AKIRA KUMAGAI,³ AND TOMOYOSHI YOSHINAGA⁴

¹National Research Institute of Aquaculture, Nansei, Mie, 516-0193, Japan; ²Tohoku National Fisheries Research Institute, Shiogama, Miyagi, 985-0001, Japan; ³Miyagi Prefectural Freshwater Fisheries Research Station, Daiwa, Miyagi, 981-3625, Japan; ⁴University of Tokyo Faculty of Agriculture, Bunkyo, 113-8657, Tokyo, Japan

ABSTRACT This experiment was designed to evaluate the genetic resistance to amyotrophy of Japanese abalone (*Haliotis discus discus*) under a mixed rearing environment. Two selected families that had previously shown resistance to abalone amyotrophy as candidates, two nonselected families as controls that had previously not shown resistance, and two hybrid families were used. Individuals from these families were fertilized and mixed immediately after hatching, and then they were raised in a mixed rearing tank at an abalone hatchery during the period of frequent spontaneous occurrences of abalone amyotrophy. Furthermore, we have isolated seven new microsatellite DNA loci for identifying families, and so the six offspring families in the mixed rearing tank could be discriminated unambiguously. The survival ratio of the two selected families was 87% and 93% after the occurrence of mass-mortality caused by abalone amyotrophy, whereas that of the four other families ranged from 0% to 37%. Survival performance among the offspring families was significantly different under the mixed rearing environment. This result suggests strongly that resistance to abalone amyotrophy of juveniles is related to the genetic characters of the spawners. Therefore, this experiment shows that resistance to the disease is a heritable trait, because the two selected families produced offspring with a high survival ratio after the occurrence of abalone amyotrophy.

KEY WORDS: abalone amyotrophy, Japanese abalone (*Haliotis discus discus*), microsatellite, parentage analysis, resistance

INTRODUCTION

Japanese abalones include four species that are economically important for commercial fisheries and as aquaculture resources in Japan, because of their high market value and their high volume of catch. However, the volume of abalone catch has been continuously decreasing for about 30 y (Anon. 2001). Therefore to increase and stabilize the commercial catch of abalone, techniques for mass production of hatchery-produced abalone seed have been developed over the last 20 y and the use of these techniques has been spread throughout the many prefectural hatcheries in Japan. In particular, the mass production of hatchery juveniles of 2 species, *Haliotis discus hannai* and *Haliotis discus discus* that support significant local fisheries, has been well studied. The juvenile production of *H. d. hannai* has increased smoothly year by year, and more than 20 million juvenile abalone were produced in 1999. However, the hatchery juvenile production of *H. d. discus* has been stagnant because mass mortalities of juvenile abalone age 0 y have frequently occurred in many abalone hatcheries, especially during the early summer when the rise in temperature is a serious problem. One of the causes of mass mortality was elucidated as an infectious disease characterized clinically with muscular atrophy and histologically with abnormal cell masses originating from nerve tissue (Nakatsugawa 1990, Momoyama et al. 1999). The disease, due to the amyotrophy of the foot muscle, has been tentatively called abalone amyotrophy. Recently, virus particles from juvenile abalone affected with amyotrophy was isolated (Otsu & Sasaki 1997, Nakatsugawa et al. 1999), however the pathogenicity of the virus has not been proved yet. Furthermore, it was reported that the mortality of juveniles spawned from one pair of parents was significantly lower than that of several other pairs during a spontaneous occurrence of abalone amyotrophy leading to mass

mortality (Okada et al. 1999). This fact suggests that the establishment of abalone families resistant to abalone amyotrophy may be possible using selective breeding.

Mixed rearing is regarded as one of the most effective methods for evaluating genetic traits, because it can minimize confounding genetic traits and environmental factors. In separate tanks, even after careful environmental standardization, environmental effects of an unknown nature are often observed to cause phenotype variation among tanks (Bagley et al. 1994, Herbinger et al. 1999). Microsatellite DNA are well known as hyper-variable genetic markers with a high discriminating power, and are regarded as an invaluable tool in the investigation of parentage analysis of hatchery populations (Herbinger et al. 1995, Blouin et al. 1996, O'Reilly et al. 1998, Perez-Enriquez et al. 1999, Hara & Sekino 2003). Discrimination of families using microsatellite DNA typing makes it possible to evaluate the genetic properties of juveniles from a mixed rearing tank.

We have developed several microsatellite DNA markers for parentage analysis in abalone to establish an evaluation method for genetic traits in mixed rearing tanks. The objective of this study is to ascertain the ability to discriminate experimental families using the developed microsatellite DNA markers and to evaluate the survival performance of the selected families resistant to abalone amyotrophy, after the spontaneous occurrence of mass mortality caused by abalone amyotrophy in the abalone hatchery.

MATERIALS AND METHODS

Six abalone families, *H. d. discus*, were fertilized artificially by pairing of three mature dams and three mature sires (Table 1). Of the six parents used, 3 parents (SD#1, SD#2, and SS#1) were the offspring from two families that had a remarkably higher survival rate than other four families among the six experimental families during an outbreak of abalone amyotrophy at Mie prefectural fish farming center in 1995 (Okada et al. 1999). Moreover, the rela-

*Corresponding author. E-mail: mhara@affrc.go.jp

TABLE 1.

Pairing of dams and sires in the six families. Relation of the dam, SD#1 and the sire, SS#1 is full-sib, and that of the dam, SD#2 and SS#1 is half sib. There is no relationship among the dam, ND#1, the sires NS#1 and NS#2.

Family	Selected		Hybrid		Non-selective	
	#1	#2	#3	#4	#5	#6
Dam	SD#1	SD#2	SD#2	SD#2	ND#1	ND#1
Sire	SS#1	SS#1	NS#1	NS#2	NS#1	NS#2

tionship between dam SD#1 and sire SS#1 was full sib, and that of dams SD#2 and SS#1 was half sib. These three parents were used for producing the candidate families resistant to abalone amyotrophy. One dam (ND1#) and 2 sires (NS#1 and NS#2) were non-selected, that is, these abalone parents were drawn randomly from the wild population. There were no relationship among ND#1, NS#1 and NS#2. The family #1 and #2, the family #3 and #4, and the family #5 and #6 were respectively called the selected, hybrid and nonselected families (Table 1). The larval offspring of the six families were mixed immediately after hatching, and were allowed to settle on plastic plates for collecting larvae in one tank. The mixed offspring (49,500 at the beginning of this experiment) were raised from November 1999 to April 2000 at an abalone hatchery of the Misaki Fisheries Co-operative Association, located in Ehime Prefecture of Japan.

To investigate the survival of abalone juveniles of the six mixed families during a spontaneous occurrence of mass mortality caused by abalone amyotrophy, 49,500 juveniles from 6–15 mm in shell length were subsequently reared from April to September 2000 in the experimental 10-ton tank of the Misaki hatchery, where mass mortality due to abalone amyotrophy has continuously occurred for several years. During the experimental rearing period, dead and moribund juveniles were removed almost every day while being observed in the experimental tank; the water temperature was measured and the number of surviving juveniles was calculated. Sampled offspring for parentage analysis were drawn randomly from the live juvenile individuals in May and August, before and after the period of mass mortality respectively. Twenty moribund juveniles were examined histologically during the occurrence of mass mortality, using a light microscope, to confirm if the mortality was caused by abalone amyotrophy.

Genomic DNA was extracted from the foot muscle tissue of the 6 parents and the sampled offspring. We have developed new hyper-variable microsatellite DNA markers for parentage analysis according to the method described in Sekino et al. (2000). Detailed information about the polymerase chain reaction (PCR) amplification protocols are found in Sekino and Hara (2001). Allele sizes were determined relative to a molecular size marker (ALFexpress Sizer 50–500, Amersham Pharmacia Biotech) in combination with the Allelelinks computer software (Amersham Pharmacia Biotech), and alleles were designated according to PCR product size. The relatedness between parents and offspring was examined by matching genotypes at each locus. Survival ratio were estimated from the percentage compositions of the families and total number of offspring reared in experimental tank.

Differences in the composition of families between before and after during mass mortality, and multiple comparisons among each family were done by χ^2 analysis, using the package Statistica (StatSoft Inc, Tokyo).

RESULTS

The estimated number of reared juvenile abalone during the experimental period is shown in Figure 1. Dead juveniles were rarely found in the experimental mixed rearing tank from April to May. After that, mass mortality occurred from June to July, and had almost ceased in August. The total estimated number of juveniles during the period from May to August decreased from 49,500 to 21,500. Decrease in feed consumption, increase in individuals with reduced activity and the appearance of abnormal growth in shell edge were observed in the experimental tank from June to July. However, these symptoms disappeared in August, and it appeared that the abnormal shell edges of individuals had recovered. During the experimental period, the temperature of the mixed rearing tank was 12.9°C to 17.5°C from April to the middle of May and was over 18°C in the end of May. After that, the temperature was continually increased reaching 23.9°C by the end of July, and staying above 24°C in August and September (Fig. 2). Furthermore, abnormal cell masses that were originating from nerve tissue and characteristic in abalone amyotrophy were histologically observed in the foot muscle section of all twenty moribund juvenile abalone, using a light microscope (Fig. 3).

We developed 7 new microsatellite DNA loci, *Hd63*, *Hd201*, *Hd306*, *Hd3105*, *Hd535*, *Hd562*, and *Hd584* from the abalone genome DNA for this experiment, and the PCR primers pertaining to them are given in Table 2. All 7 loci were successfully amplified for 6 abalone parents, and the genotypes of the 6 abalone parents for the 7 microsatellite DNA loci and the number of alleles per locus are listed in Table 3. The number of alleles per locus ranged from 6 to 8 with an average of 6.7, and alleles unique to one parent were found at all loci. Further, all the parents except SD#1 had unique alleles. These microsatellite DNA loci were hyper-variable and stable to analysis.

The parents of individual offspring could be discriminated unambiguously by observations of the unique alleles and/or by comparison of the multi loci genotype for the 7 microsatellite DNA. The parentage inferred from more than two loci agreed perfectly for all analyzed individuals. The families of all offspring were determined precisely by the relatedness between parents and offspring. The percentages and individual number of offspring related to 6 families in May and those of August are shown in Table 4.

The estimated numbers of the selected families (family #1 and

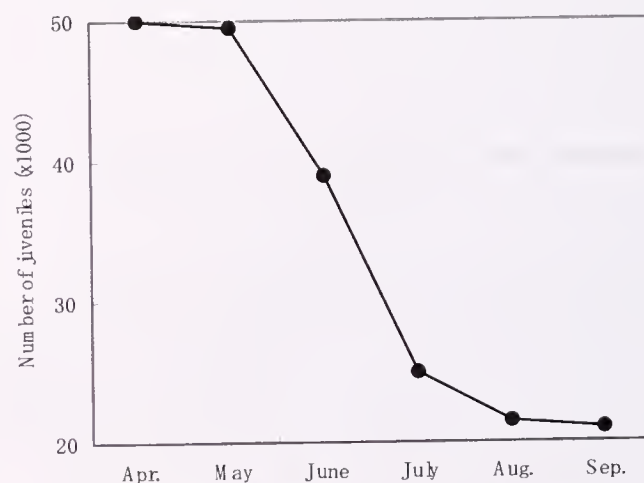


Figure 1. Number of surviving juveniles during the experimental rearing period from April to September.

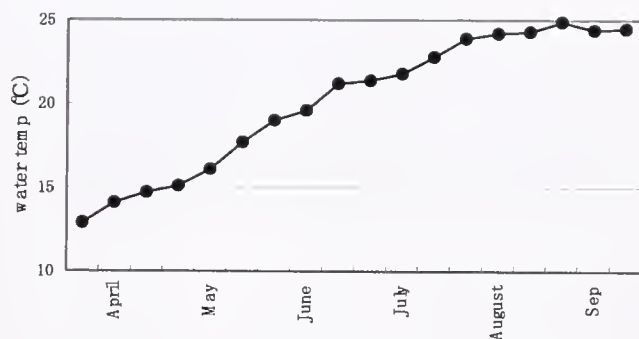


Figure 2. Changes of water temperature from April to September in this experimental mixed rearing tank.

#2) were 38 and 22 of the 236 analyzed individuals, that is 16.1% and 9.3% respectively of the total mixed families in May, and the percentage compositions of these families had proportionally increased to 34.6% and 18.7% respectively in August. However, the percentage composition of the two hybrid and two nonselected families (family #3, #4, #5, and #6) decreased from May to August. Especially, offspring of the families #6, which (15.3%) was 36 of the 236 analyzed individuals, were not found at all in August. The percentage composition of the 6 families in May was significantly different compared with that in August ($P < 0.01$). Furthermore, on the multiple comparisons among individual number of each family in May and August, significant differences were recognized between the two selected families (family #1 and 2) and the other four families and between the family #6 and the other families ($P < 0.05$). The estimated survival ratio of the selected two families (family #1 and #2), which was calculated based on the total number of offspring reared in the tank and the percentage composition of each family, was more than 80%. However, the estimated survival ratios of the other families were less than 40%.

DISCUSSION

Mass mortality occurred spontaneously from June to July in the experimental mixed rearing tank at the abalone hatchery, and more than half of the juvenile abalone died. This period of mass mortality coincided with the season of mass mortalities reported previously (Nakatsugawa 1990, Momoyama et al. 1999, Okada et al. 1999). Momoyama et al. (1999) histologically examined individual abalone from mass mortalities that occurred in different hatcheries during this period from 1988 to 1996, and they reported that most of mass mortalities were attributable to abalone amyotrophy. The characteristic relation between the occurrence of abalone amyotrophy and ambient water temperature has been reported as follows: the progress of abalone amyotrophy occurs when the water temperature rises to 18°C or higher and is suppressed at more than 23°C (Nakatsugawa 1990, Okada et al. 1999). The range of water temperature of this experimental mixed rearing tank during this mass mortality was consistent with the typical temperature range of mass mortality caused by amyotrophy disease. Unhealthy individual juveniles and with shell edge abnormalities appeared in the experimental tank during this mass mortality period. These symptoms coincide with the characteristics of abalone amyotrophy. Furthermore, abnormal cell masses that were previously reported as characters of abalone amyotrophy (Nakatsugawa 1990, Momoyama et al. 1999) could be observed from all individuals examined histologically. These findings

strongly suggest that the mass mortality during this experimental rearing period was caused by abalone amyotrophy.

The usefulness of microsatellite DNA markers for parentage determination in hatchery populations has been previously reported (Herbinger et al. 1995, Perez-Enriquez et al. 1999, Hara & Sekino 2003). In this experiment, the parent-offspring relationships could be unequivocally identified for all abalone offspring mix-reared from the larval stage onward using the seven new microsatellite DNA loci, and the families of the offspring were accurately discriminated. As a result, the change of the percentage composition of the six families from May to August suggests that the survival of the 2 selected families (family #1 and #2) were significantly better than that of the other 4 families (family #3, #4, #5, and #6) after the occurrence of mass mortality caused by abalone amyotrophy. Juvenile individuals of the six families were reared in the same tank, thus minimizing environmental effects immediately after hatching; therefore their survival performances among families are considered to have been strongly influenced by heritability.

The two selected families were produced from the families that had higher survival rates than the other families after an occurrence of mass mortality caused by abalone amyotrophy, and in this experiment their offspring had survived higher rates than the other families examined. This result suggests that resistance to abalone amyotrophy of the selected families is a heritable trait. However, the estimated survival ratios of the 2 hybrid families (family #3 and #4) that were produced by the dam of the candidate families tolerant to the disease did not differ from that of the one nonselected family (family #5). The family of spawners used as tolerant to abalone amyotrophy in this experiment might not be perfectly fixed genetically. Differences between families for survival ratios in NS#1 and NS#2 of nonselected sires indicate the existence of various degrees of tolerance to abalone amyotrophy in wild abalone populations.

The results of this experiment strongly suggested that resistances to abalone amyotrophy is due to genetic effects. Establishment of a method of experimental infection by isolation of the pathogen of abalone amyotrophy and development of high performance families resistant to the disease, using selective inbreeding techniques, would likely help the problem of mass mortality caused by amyotrophy disease for juvenile production of Japanese abalone, *Haliotis discus discus*.

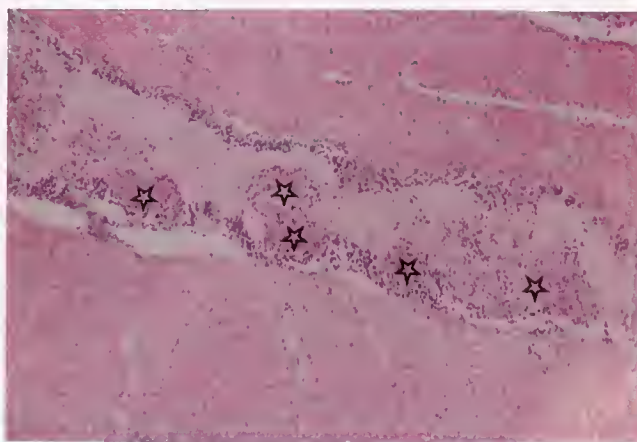


Figure 3. Muscular section of moribund juvenile abalone during the occurrence of mass mortality. Abnormal cell masses like tumors (—) are observed in muscles organs.

TABLE 2.

Core microsatellite sequences, primer sequences, and annealing temperatures (T_m) for seven microsatellite DNA loci of Japanese abalone *Haliotis discus discus* used in this experiment.

Locus	Core Repeat Sequence (5'-3')	Primer Sequence (5'-3')	T _m (°C)	GenBank Accession No.*
<i>Hd63</i>	(CA) ₁₆	F-CACTATATAAATGCGGCATAAG R-GCTTTGTAAAGTGCAGTAATC	50	AB025376
<i>Hd201</i>	(CA) ₅ (CG) ₂ (CGCA) ₃ (CG) ₂ (CGCA) ₆ CT(GCACC) ₃ GCACG(CA) ₈	F-CTTTTATGAATATGCGATTTCCTGA R-TCACTCTGTGAAGGTTTCACTCCA	61	AB085642
<i>Hd306</i>	(CA) ₃ CAC(CA) ₇ (CCA) ₂ (CA) ₁₉ C (CA) ₃ CX ₄ (AC) ₄ G(CA) ₄ X(CA) ₆	F-GGAACAGTTTACAAGGTGGGAGCA R-GGTTTGTTCACAGGCCGCCATCGC	64	AB085643
<i>Hd3105</i>	(CA) ₅ (CAG) ₂ TA(CGCA) ₃ (CG) ₂ C (CAG) ₆ (CATG) ₂ (CA) ₆	F-GTTGTAATGGTGAATCGGAC R-CACTAACGTAGTGAGGTGCA	50	AB085644
<i>Hd535</i>	(CACT) ₁₄	F-TTAACTCTACATGCCGAAG R-TACTGTCAGTCCACATAGGAT	50	AB085645
<i>Hd562</i>	(TG) ₂ (TTG) ₃ (TCG) ₂ (TTG) ₇	F-TGGTTGTGGCCTTGCTGTTTC R-TATAGCTGGAATGCTCAGTGCG	63	AB085646
<i>Hd584</i>	(ACTC) ₁₇	F-TATGACGGGAATATTGCTAA R-CAAAATGTGGTTAACATAGATAT	51	AB085647

* The nucleotide sequence data will appear in the DDJB/EMBL/GenBank nucleotide databases with the accession numbers.

TABLE 3.

Genotypes of the 6 parents at the 7 microsatellite DNA loci in Pacific abalone, *Haliotis discus discus*. Block characters indicate unique alleles.

Locus	Dam			Sire			Numbers of Alleles in Parents	Numbers of Unique Alleles
	SD#1	SD#2	ND#1	SS#1	NS#1	NS#2		
<i>Hd63</i>	176/176	170/172	178/180	172/176	170/184	172/172	6	3
<i>Hd201</i>	200/222	190/222	196/218	200/222	196/224	174/212	8	5
<i>Hd306</i>	256/296	270/270	226/226	256/296	226/226	194/242	6	3
<i>Hd3105</i>	218/252	218/226	216/224	218/252	220/220	266/266	7	4
<i>Hd535</i>	175/211	183/195	175/175	187/211	175/199	175/195	6	3
<i>Hd562</i>	163/180	163/169	159/169	163/173	171/187	169/173	8	3
<i>Hd584</i>	131/131	131/131	135/139	131/131	115/143	115/119	6	3

TABLE 4.

Estimated number of offspring and survival ratio of six families reared in tank during the occurrence of mass mortality from May to August. Numbers of offspring (N_t) are estimated based on the genetic markers of microsatellite DNA. Number in parentheses are the percentage of offspring of each family. N(M) and N(A) means the estimated actual number of offspring of families in the tank in May and August based on the percentage respectively.

Family (♀ × ♂)	Estimated Number of Offspring				Estimated Survival Ratio (%) *1 (N(A)/N(M) × 100)
	May N _t (%)	N (M)	August N _t (%)	N (A)	
#1 (SD#1 × SS#1)	38 (16.1)	7970	37 (34.5)	7418	93 ^a
#2 (SD#2 × SS#1)	22 (9.3)	4604	20 (18.7)	4021	87 ^b
#3 (SD#2 × NS#1)	56 (23.7)	11732	22 (20.6)	4429	38 ^c
#4 (SD#2 × NS#2)	27 (11.4)	5643	6 (5.6)	1204	21 ^d
#5 (ND#1 × NS#1)	57 (24.2)	11979	22 (20.6)	4429	37 ^c
#6 (ND#1 × NS#2)	36 (15.3)	7574	0 (0)	0	0 ^c
Total number of offspring analyzed	236 (100)		107 (100)		
Total number of offspring reared in the tank		49500		21500	43

* 1 Values with different superscripts indicate significant differences ($P < 0.01$).

ACKNOWLEDGMENT

The authors thank Dr. Katsuhiko Wada, National Research Institute of Fisheries Science and Dr. Takuji Okumura, National Research Institute of Aquaculture for helpful suggestions and advice of statistical analysis. The authors also thank Dr. Mamoru Nishimura, Mie Prefectural Fisheries Experimental Station for the

donation of the candidate abalone resistant to abalone amyotrophy and Mr. Shogo Kikuchi and Mr. Kondo, Misaki Fisheries Co-operative Association for rearing the abalone seed and supplying the samples. This study was supported in part by a grant-in-aid (Development fundamental technologies for effective genetic improvement of aquatic organisms program) from the Ministry of Agriculture, Forestry and Fisheries, Japan.

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VIRUS INFECTION IN CULTURED ABALONE, *HALIOTIS DIVERSICOLOR* REEVE IN GUANGDONG PROVINCE, CHINA

JIANGYONG WANG,^{1,2} ZHIXUN GUO,¹ JUAN FENG,¹ GUANGFENG LIU,¹ LIWEN XU,¹ BISHENG CHEN,^{1*} AND JINPEI PAN²

¹South China Sea Fisheries Research Institute, Guangzhou 510300, People's Republic of China;

²South China Sea Institute of Oceanology, The Chinese Academy of Sciences, Guangzhou 510301, People's Republic of China

ABSTRACT A serious disease occurred in many *Haliotis diversicolor* Reeve farms during 1999 and 2002. The symptoms of the infected abalone were as follows: the pleopod became stiff, its surface became black, and the mantle shrank. All sizes of abalone became infected and mortality was high. A spherical virus was detected in the tissues of the digestive gland, mantle, gill, and intestine of infected animals. It was observed in the cytoplasm and had a diameter about 100–130 nm. Electron microscope examination showed that organelles of infected cells, such as the endoplasmic reticulum; expanded, mitochondria dilated, nuclear membranes dissolved or disappeared, and the edges of the nucleolus swelled. The pathogenicity of the virus was confirmed by experimental infection.

KEY WORDS: virus infection, pathogen, *Haliotis diversicolor* Reeve

INTRODUCTION

Numerous pathogens that cause serious abalone diseases have been reported from different abalone species around the world. Virus-like particles have been observed in the cytoplasm of cells near the nerve trunk of diseased *Haliotis discus discus*, examined with an electron microscope (Otsu & Sasaki 1997). Virus-like particles were also isolated from diseased *H. discus discus* in primary cultures of abalone hemocytes (Nakatsugawa et al. 1999). A spherical virus was detected in "crack shell disease" of *Haliotis discus hannai* (Wang & Li 1997, Li et al. 1998). A similar disease and virus in *Haliotis diversicolor* Reeve were also studied (Wang et al. 2000). The pathogen of an epidemic disease that resulted in mass mortality of *Haliotis diversicolor aquatilis* was studied. Artificially infected abalone revealed that the virus was the lethal pathogen (Song et al. 2000). Three forms of spherical viruses were observed when an epidemic disease of cultured *Haliotis diversicolor supertexta* occurred in Dongshan, Fujian province in 1999 (Zhang et al. 2001).

A bacterial infection was reported in juveniles of red abalone *Haliotis rufescens* (Elston & Lockwood 1983). Mass mortalities of 4-day-old red abalone juveniles were caused by *Vibrio alginolyticus* (Anguiano-Beltrán et al. 1998). A disease of young *Haliotis diversicolor supertexta* was also caused by *Vibrio* spp. The abalone were more easily infected when the temperature was higher (Lee et al. 2001). Other bacteria that have been isolated from *Haliotis discus hannai* are *Vibrio fluvialis*-II (Liu et al. 1995, Nie et al. 1995, Li et al. 1996), *V. campbellii* (Ma et al. 1996), and *Pseudomonas fluorescens* (Ye et al. 1997). Parasites that infect different species of abalone include ciliates in *H. midae* and *H. spadicea* (Botes et al. 1998), kidney Coccidia in *Haliotis rufescens* and *Haliotis midae* (Mouton 2000, Sales & Britz 2000), *Perkinsus* spp. in *Haliotis rubra*, *Haliotis laevigata* (Goggin & Lester 1995), sabellid polychaetes in *Haliotis midae* (Cook 1998, Ruck & Cook 1998, Sales & Britz 2000), and trematode metacercariae in *Haliotis ruber* (Botes et al. 1999).

Haliotis diversicolor Reeve has been increasingly cultured along the southern coastal area of China and has become one of the

most exploited and valuable mariculture species. Mass mortalities have been observed in many commercial farms since a serious disease occurred. All sizes of abalone could be infected, and the mortality may reach 100% in a few days. The culture tanks were filled with foam. The pleopod of the diseased abalone became stiff with a black surface and the mantle and foot shrank. The dead abalone attached to the bottom of the culture cage. Here we study the pathogen, the influence of temperature on its infectivity, and the ultrastructure pathology of the infected abalone.

MATERIALS AND METHODS

Specimens

Diseased abalone, of shell length 4–6 cm, were collected from several commercial farms located in the eastern area of Guangdong Province during 2000–2002.

Healthy abalone, of shell length 4–6 cm, were collected from a farm located in an isolated bay that had never experienced the same disease.

Preparation of Solution for Injection

Ten grams of tissue of diseased abalone was cut into small pieces and homogenized, using a mortar, in the presence of liquid nitrogen. The homogenate was suspended with phosphate-buffered saline (PBS) (pH = 7.6) (W: W = 1:10) and the suspension was centrifuged at $\times 2500g$ for 30 min at 4°C. The supernatant fluid was centrifuged at $\times 6000g$ for 30 min at 4°C, and the supernatant fluid was collected and filtered through a 0.22- μm filter membrane.

The suspension from the healthy abalone was prepared using the same procedure.

Challenge Test

Four groups were used for challenge test 1, and were treated as indicated in Table 1. The animals in group 1 were injected with 0.05 mL virus suspension each; group 2 were injected with 0.1 mL virus suspension each; the animals in control group 1 were injected with 0.1 mL suspension of healthy abalone each; the animals in control group 2 were injected with 0.1 mL PBS each. All animals were maintained in the aquaria with recirculating water system at

*Corresponding author. E-mail: chenbs309@163.com.

TABLE 1.

Preparation of challenge test in *halotis diversicolor* Reeve.

Group	Number of Healthy Abalones	Dose of Injection	Temperature for Maintaining
Group 1	10	0.05 ml virus suspension	17°C
Group 2	10	0.1 ml virus suspension	17°C
Control Group 1	10	0.1 ml suspension of healthy abalone	17°C
Control Group 2	10	0.1 ml PBS	17°C

17°C. The salinity of the water was 30 ppt. The mortality and clinical signs were observed and recorded every day.

Temperature Influence on Disease Outbreak

The abalone were divided into 5 groups with 10 individuals each. They were treated as indicated in Table 2. The animals in group 3, group 4, group 5, and group 6 were injected with 0.1 mL virus suspension respectively; the animals in control group 3 were injected with 0.1 mL PBS. All animals were maintained in aquaria with recirculating water, the salinity of the water was 30 ppt. Mortality and clinical signs were observed and recorded every day.

Transmission Electron Microscopy

The tissues of digestive gland, mantle, gill, and intestine of both naturally and experimentally infected abalone were cut into pieces about 1 mm³ in size, and fixed in 2.5% glutaraldehyde in 0.1 PBS buffer at 7.4. After being washed in PBS buffer for 1 h at 4°C, samples was postfixed in 1% osmium tetroxide in the same buffer at 4°C. The samples were dehydrated in ascending concentrations of ethanol (50% to 100%), and then embedded in Epon812 resin. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate, and examined in a PhilipsCM10 transmission electron microscope.

Negative Stain

Twenty grams of tissue of diseased abalone was cut into small pieces and homogenized using a mortar, in the presence of liquid nitrogen. The homogenate was suspended in phosphate-buffered saline (PBS) (W: W = 1:10) and the suspension was centrifuged at ×6000g for 30 min at 4°C. The supernatant fluid was centrifuged at ×200000g for 60 min at 4°C, and the deposit was resuspended with PBS and stained with 2.5% sodium phosphotungstate (pH 7.0), and then examined in a Philips CM10 transmission electron microscope.

TABLE 2.

Temperature influence on disease outbreak.

Group	Number of Healthy Abalones	Dose of Injection	Temperature for Maintaining
Group 3	10	0.1 ml virus suspension	17°C
Group 4	10	0.1 ml virus suspension	20°C
Group 5	10	0.1 ml virus suspension	23°C
Group 6	10	0.1 ml virus suspension	26°C
Control Group 3	10	0.1 ml PBS	17°C

RESULTS

Challenge Test

Several dead abalone were observed in group 1 and group 2 that were injected with virus suspension within 48 h and all died within 96 h (Table 3). The aquarium was filled with foam, which also occurred when the infection occurred naturally (Fig. 1a). The clinical symptoms of infected abalone were also similar to naturally infected abalone (Fig. 1b). No mortality was recorded in the control groups.

Temperature Influence on Disease Outbreak

The disease outbreak was associated with low temperature (Table 4). All the abalone died within 96 h when the temperature of the water was at 17°C and 20°C. No abalone died when the temperature of water was 23°C or 26°C. All abalone in the control group survived.

Electron Microscopy

Infected cells in the connective tissue showed hypertrophied or irregular shaped nuclei and margined chromatin (Fig. 1c, d), which indicated the condensation of chromatin and DNA. Some nuclei were highly electron dense. Chromatin was densely packed around the nuclear envelope, and the membrane of the nucleus disintegrated (Fig. 1c). The nucleus collapsed in seriously affected cells. The nuclear membrane totally dissolved, and some highly electron dense material could be seen in the collapsed nucleus (Fig. 1e).

Pathologic changes were observed in the organelles of the infected cells. The endoplasmic reticulum expanded, mitochondria dilated and became irregularly shaped, and mitochondrial cristae shrank or disappeared (Fig. 1f). In the cytoplasm, irregularly shaped spore-like bodies were observed. The bodies appeared to be mineral concretions, composed of numerous alternating electron dense and electron lucent layers (Fig. 2a).

Virus particles were mainly observed in connective tissue cells and occasionally in hemocytes. The particles were circular in shape, 100–130 nm in diameter (Fig. 2b), contained an electron-dense nucleocapsid, and the outer layer was the thick envelope. Between the envelope and nucleocapsid, there was an electron lucent layer about 10–15 nm in thickness. The nucleocapsid was electron-dense and 80–100 nm in diameter. In some cells, numerous virus particles aggregated together and were surrounded by a double-layer membrane (Fig. 2c, d). The virus particles scattered in the cytoplasm in some infected cells (Fig. 2e, f). Figure 3a shows a magnification of the virus particles. Among the aggregat-

TABLE 3.

The cumulative mortality of challenge test.

Group	Time (h)				Mortality Rate
	24	48	72	96	
Group 1	0	2	7	10	100%
Group 2	0	3	9	10	100%
Control Group 1	0	0	0	0	0
Control Group 2	0	0	0	0	0

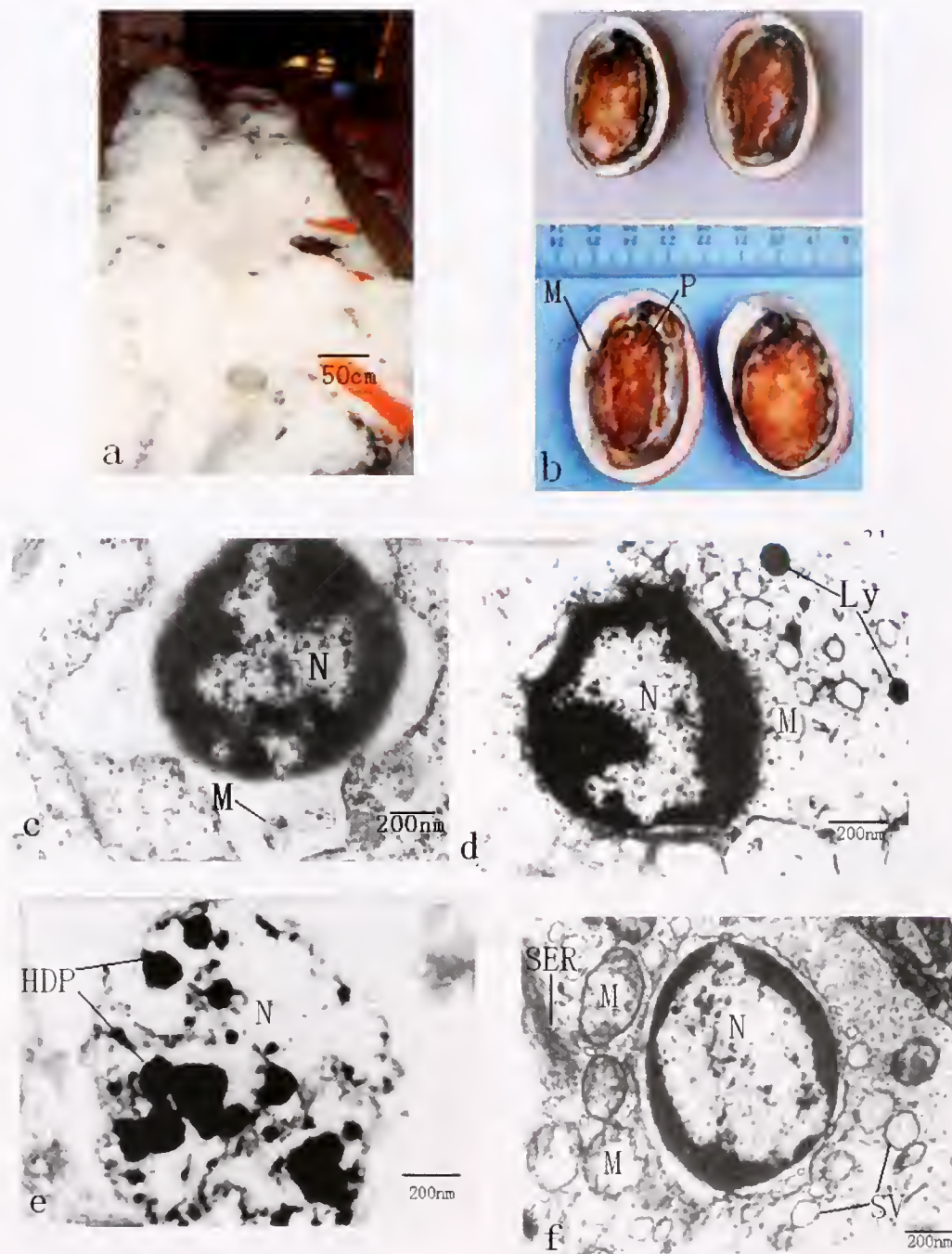


Figure 1. a. The tank in which disease occurred filled with foam. b. Infected abalone with shrunken mantle and pleopod, M = mantle; P = pleopod. c. Infected cells with hypertrophied nuclei and margined chromatin, nuclear membrane disintegrated. M = mitochondrion; N = nucleus. d. Infected cells with hypertrophied nuclei and margined chromatin, organelle dissolved. Ly, Lysosome; M = mitochondrion; N = nucleus. e. Collapsed nuclear, N = nucleus; HDP, highly dense particle. f. The pathologic changes of organelle of infected cell, endoplasmic reticulum expanded, mitochondria dilated with irregular shape, nuclear membranes dissolved or disappear, the edges of nucleolus swelled. M, mitochondrion; N = nucleus; SER = smooth endoplasmic reticulum; SV, small vesicle.

ing viruses, there were empty and deformed capsid-like structures (Fig. 2c). These may be abnormally assembled viral proteins. TEM examination revealed that virus particles existed in challenged individuals, but not in nonchallenged ones.

Negative stain showed the virus particle was about 100–130 nm in diameter (Fig. 3b), similar to the size of virus particle observed in ultra-thin section (Fig. 3a).

DISCUSSION

Virus are some of the most important agents that cause mass mortalities of animals. Viruses reported in marine shellfish include Herpesviridae, Iridoviridae, Papoviridae, Togaviridae, Retroviridae, Reviridae, and Paramyxoviridae.

Several viruses associated with the mass mortalities of the host

TABLE 4.
The cumulative mortality of infected abalones at
different temperature

Group	Time (h)				Mortality Rate
	24	48	72	96	
Group 3	0	2	7	10	100%
Group 4	0	4	8	10	100%
Group 5	0	0	0	0	0
Group 6	0	0	0	0	0
Control Group 3	0	0	0	0	0

species have been reported. Irido-like virus attacked the velar epithelium of *Crassostrea gigas* and caused severe hatchery losses (nearly 100%), which usually appeared from March to May, but also occurred throughout the summer (Elston, 1979). Icosahedral DNA virus caused the Portuguese oyster *Crassostrea angulata* Lamarck velar virus disease and hemocyte infection virus disease (HIV), extensive gill erosion corresponding with high mortalities. Gill disease was regarded as one factor in the elimination of the Portuguese oyster from important culture areas on the Atlantic coast of France (Comps 1988). In *Tiostrea chilensis* larvae and spat, the interstitial cells, mantle, and digestive tract of epithelial cells were infected with herpes-like viruses and infection seemed to be associated with about 95% mortality, over 3 to 4 days, among experimentally exposed veligers (Hine et al. 1998). Herpes-like viruses were the main agents that led to high mortalities of the

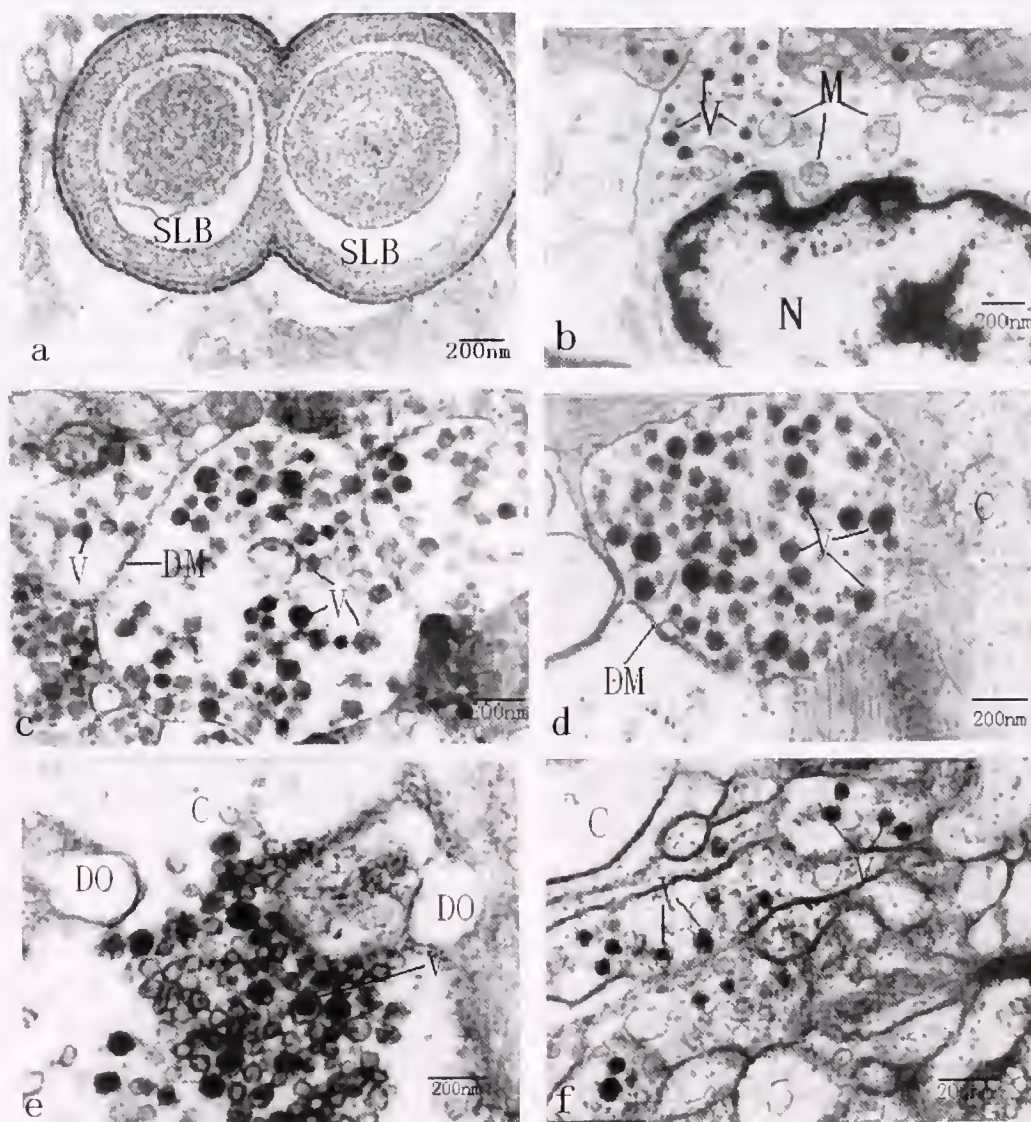


Figure 2. a. Spore-like bodies appeared to be mineral concretions composed of numerous alternating electron dense and electron lucent layers. C = cytoplasm, SLB = spore-like body. b. Infected cell, mitochondrion dilated, M, mitochondrion; N = nucleus; V = virus particle. c. Virus particles existed either inside of or outside of the double-layer membranes. DM = double membranes; V = virus particle. d. Virus particles enveloped in the double membranes, DM = double membranes; V = virus particle. e. Virus particles scattered in the cytoplasm of the infected cell. C = cytoplasm; DO = dissolved organelle; V = virus particle. f. Virus particles scattered in the cytoplasm of the infected cell. C = cytoplasm; V = virus particle.

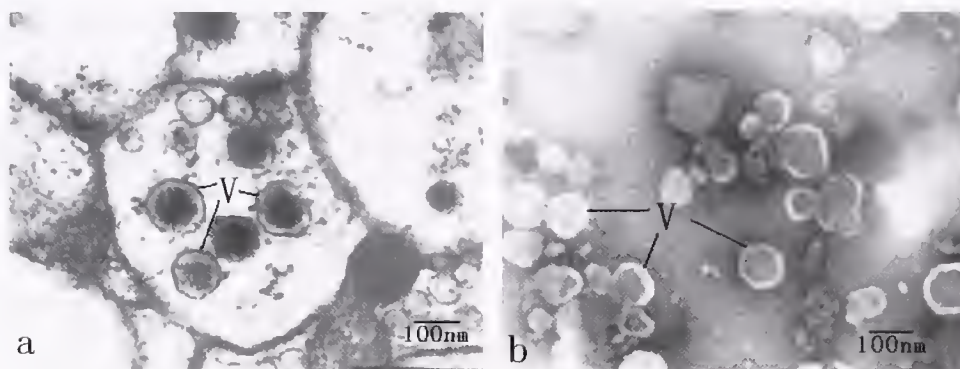


Figure 3. a. The magnification of the virus particles. V = virus particle. b. The negative stain showed the virus particles. V = virus particle.

Pacific oyster *Crassostrea gigas* larvae (Renault et al. 2001). The herpes-like viral infection in larval *Ruditapes philippinarum* was associated with sporadic high mortalities in a commercial hatchery (Renault et al. 2001). Electron microscopic examination revealed the presence of spherical virus-like particles in the digestive gland, kidney, and intestine of moribund scallop *Chlamys farreri* in 2000. The virus was approximately 130–170 nm in diameter and always observed in the cytoplasmic vesicles of infected cells. It was the main cause of the mass mortality of scallops, from July to August since 1997, in the northern area of China (Liu 2002).

With the development of abalone culture industries, the impact of diseases on abalone have become more and more serious. We described, for the first time, the virus infection in abalone *Haliotis diversicolor* Reeve associated with the mass mortalities in Guangdong Province. With respect to the size, morphologic features, and syndrome of the infected abalone, it was similar to the 100 nm diameter spherical virus that was found in the cytoplasm of liver cells of diseased *Haliotis diversicolor* Aquilatis from Fujian Province (Song et al. 2000). In our study, the virus particles were mainly observed in the cytoplasm of the infected cells, but never in the nucleus.

Pathologic changes in infected cells included expanded endoplasmic reticulum and dilated or dissolved mitochondria. In the cytoplasm, the endoplasmic reticulum is associated with the Golgi body and mitochondria and functions in synthesis, transportation, and secretion (Wu Zh 1990). The impact of viruses on cells included the destruction of membrane structures of organelles such as endoplasmic reticulum and mitochondria. The viruses were enveloped by the membrane systems of these organelles (Fig. 2d).

The disease infected all sizes of abalone when the temperature was $<20^{\circ}\text{C}$. It seemed to break out more easily when the temperature became lower. When the water temperature was at $\geq 23^{\circ}\text{C}$, mortality did not occur. It is suspected that the viruses propagate only in low temperatures. The optimal temperature for *Haliotis diversicolor* Reeve to grow and develop is about 25°C , and it may be that physiologic changes that occur in the species at lower water temperatures may make the animals more susceptible to infection.

ACKNOWLEDGMENTS

This research was supported by the Department of Science and Technology of Guangdong Province. The project number is 2KB05301N.

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A NEW SPECIES OF *CRYPTOBIA* SP. N. (KINETOPLASTIDA, BODININA, BODONIDAE) FOUND IN THE BLOOD OF THE FARMED ABALONE, *HALIOTIS DIVERSICOLOR* REEVE

BISHENG CHEN,^{1,*} LIWEN XU,¹ ZHIXUN GUO,¹ AND HONGZHI YANG²

¹South China Sea Fisheries Research Institute, CAFS, People's Republic of China; ²Longgang District Fisheries Research Institute, Shenzhen, People's Republic of China

ABSTRACT A new species of *Cryptobia*, was found in the blood of diversicolor abalone, *Haliotis diversicolor* Reeve, derived from farming ponds, on the coast of the south China sea in December 2000. Stained with Giemsa smear, most organisms are spindle-shaped, $12.5 \pm 1.88 \mu\text{m}$ in length, $2.50 \pm 0.85 \mu\text{m}$ in width ($n = 20$), and the nucleus is $1.81 \pm 0.61 \mu\text{m}$ in length, and 1.22 ± 0.33 in width. Two unequal flagella are derived from a flagella pocket. The kinetoplast is mostly single, located anterior to the nucleus. Transmission may be direct, from one host to another through lesions on the skin or gill, or it may be also by a vector.

KEY WORDS: parasite, *Cryptobia* spp, new species, *Haliotis diversicolor* Reeve

INTRODUCTION

Since the early 1990s, epizootic mortalities have been observed in many sea-board farmed discus abalones *Haliotis discus hannai*, and diversicolor abalone, *H. diversicolor* Reeve in China. There are several varied pathogenetic organisms found in cultured abalones, including virus (Li et al. 1998, Nakatsugawa et al. 1998, Wang et al.2000, Fang et al.2002), bacteria (Li et al. 1996, Liu et al.2000, 2001, Lee et al. 2001), parasites (Bower et al. 1989, Gogging & Lester 1995, Ruck & Cook 1998, Ruck & Cook 1999, Chen et al. 2001) and rickettsiae-like organisms (Moore & Robbins 2000, Chen et al. 2001), and the like. A dinoflagellate has been found in blood of pathogenic abalones, *H. diversicolor* Reeve. This study describes the structure of the parasite in farmed abalones on the coast of south China.

*Corresponding author. E-mail: chenbs309@163.com

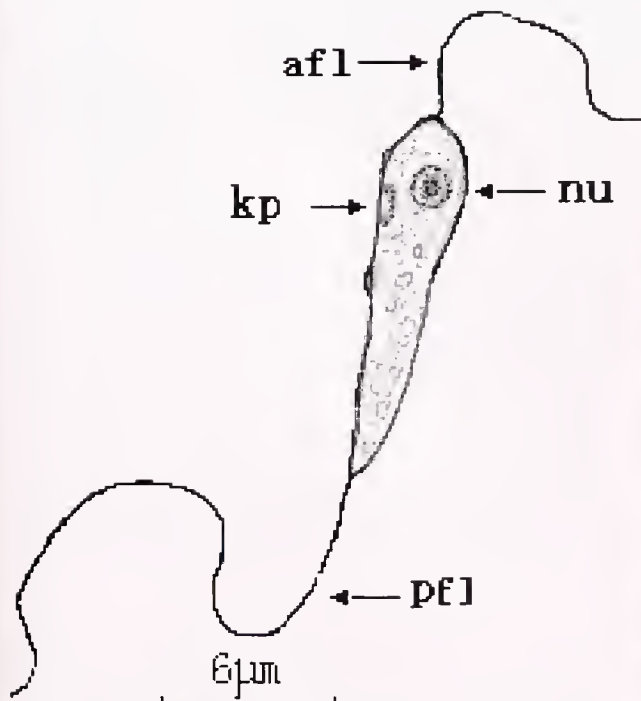


Figure 1. *Cryptobia* sp. Mode picture. afl, anterior flagellum; kp, kinetoplast; nu, nucleus; pfl, posterior flagellum.

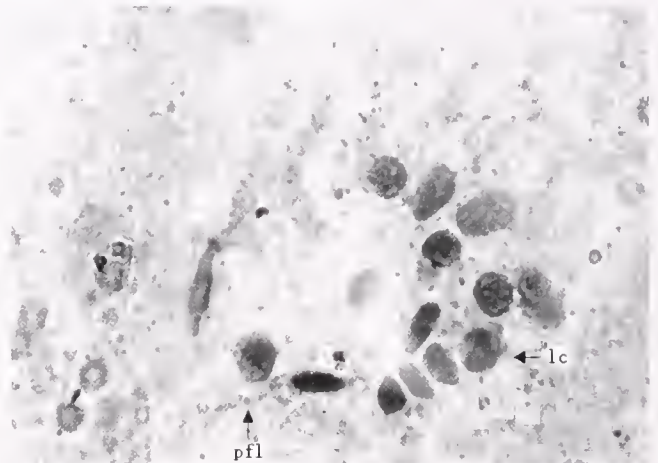


Figure 2. *Cryptobia* sp. in lymph blood of abalone. Stained with Giemsa smear, $\times 400$. lc, lymph-cell pfl: posterior flagellum.

MATERIALS AND METHODS

Cultured diversicolor abalones with a mean length of 680 mm (580–720 mm), and mean weight of 20 g, were obtained occasionally from 4 farming ponds in Shanwei, Guangdong Province. After dissection, inhalant lymph blood of abalone was obtained from the

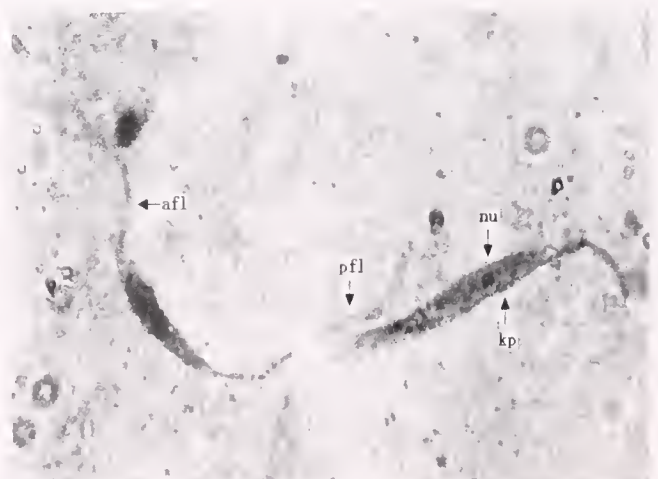


Figure 3. *Cryptobia* sp. stained with Giemsa smear, $\times 1000$. afl, anterior flagellum; kp, kinetoplast; nu, nucleus; pfl, posterior flagellum.

TABLE 1.
The comparison of two haemoflagellates.

Parasites	Hosts	Body Length × Width (μm)	Anterior Flagellum (μm)	Posterior Flagellum (μm)	Situated nucleus Length × Width (μm)	Kinetoplast	Source
<i>Cryptobia chongqingensis</i>	Misgurnus anguillicandatus	52.82 × 7.9	42.8 × 2.37	—	6.93 × 2.37	Bar-shaped	Zhao and Ma 1992
This <i>Cryptobia</i> sp.	<i>Haliotis diversicolor</i>	(12.5 ± 1.88) × (2.5 ± 0.25)	8.85 ± 1.23	15.38 ± 2.46	(1.81 ± 0.61) × (1.22 ± 0.33)	Ellipsoidal with a endosome	This paper

Cryptobia sp. nov.

Synonym, *Cryptobia abalonesis* Chen 2001; Host and site, (Gastropod) *Haliotis diversicolor* Reeve, bloodstream of lymph sinus; Locality and date, Seaboard farms of Shanwei, Guangdong Province, December 2000; Depositions, Type specimens and slides will be deposited in the South China Sea Fisheries Institute, CAFS, China, and in the collection of the authors.

heart and then smeared on to slides. Smears were fixed in methanol for about 5 min, stained with Giemsa's fluid diluted 1:10 with water at pH 7.0–7.2 PBS for about 30 min, then rinsed well in distilled water. Results were observed microscopically.

RESULTS

Most of detected abalones ($n = 20$) were infected by parasites. The parasite density was 2–8 *Cryptobia* sp. in every view ($\times 400$) (Fig. 1 and Fig. 2). The organisms are spindle shaped, $12.5 \pm 1.88 \mu\text{m}$ in length, and $2.5 \pm 0.85 \mu\text{m}$ in width. The nucleus is ellipsoidal with an endosome, situated at 20% to 25% of body length from anterior extremity. The nucleus is $1.81 \pm 0.61 \mu\text{m}$ in length, and $1.22 \pm 0.33 \mu\text{m}$ in width. The kinetoplast is mostly single spherical, located anterior to the nucleus. Two unequal flagella are derived from a flagella pocket. The anterior flagellum, $8.85 \pm 1.23 \mu\text{m}$ in length, is shorter than the posterior one ($15.38 \pm 2.46 \mu\text{m}$). The undulating membrane is not well developed and forms a narrow undulating membrane when stained with Giemsa smear (Fig. 3).

DISCUSSION AND CONCLUSION

The flagellates mostly are pathogenic protozoa that cause disease and mortality in freshwater and seawater hosts. They have been often found in the gills (Lom & Dykova 1992, Diamant

1990), epidermis (Kreier 1977, Kinne 1980), intestinal tract (Kozloff 1948), as well as bloodstream (Chen & Hsieh 1964, Zhao & Ma 1992). A species appearing identical to *Cryptobia helices* has also been reported living in the reproductive organs and intestines of pulmonate land snails in Europe (Leidy 1846). In this investigation a new hemoflagellate (*Cryptobia* sp.) occurred firstly in the blood of abalone. It differs from other described species in its shape and body size, as well as the shape of the nucleus and flagellum (Table 1).

In recent years, Cryptobiosis, caused by the hemoflagellate, has been found in many fishes (Lom & I. Dykova 1992, Kreier 1977, Kinne 1980). The use of monoclonal antibody probes and immuno-chemotherapeutic strategies have been reported to detect the pathogenic hemoflagellate (Lukes et al. 1998, Ardelli & Woo 2001). There are few effective chemotherapies against the parasite in intensive abalone culture facilities. It is, therefore, necessary to develop a sensitive detection technique and an effective control method against this parasitic organism.

ACKNOWLEDGMENTS

This work was supported by Department of Science and Technology of Guangdong Province, PRC., under grant No. 2KB05301N.

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RESPONSE OF INNATE IMMUNE FACTORS IN ABALONE *HALIOTIS DIVERSICOLOR SUPERTEXTA* TO PATHOGENIC OR NONPATHOGENIC INFECTION

WANG SHUHONG,¹ WANG YILEI,¹ ZHANG ZHAOXIA,² RALPH JACK,³
WENG ZHAOHONG,¹ ZOU ZHIHUA,¹ AND ZHANG ZIPING^{1,*}

¹Fisheries college, Jimei University, Xiamen, 361021, China; ²Oceanography department, Xiamen University, Xiamen, 361005, China; ³Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand; ⁴Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI 48824

ABSTRACT Cell free hemolymph from *Haliotis diversicolor supertexta* was prepared from fluid collected at 1, 4, 8, 12, 24, 48, 96 h after injection with either *Escherichia coli*, *Vibrio parahaemolyticus* or 0.9 NaCl solution (control group). The response of selected innate immune parameters (lysozyme, antibacterial activity, alkaline phosphatase, acid phosphatase, phenoloxidase, and superoxide dismutase) was investigated. Results showed that the activities of ACP (Acid Phosphatase) from abalone injected with *V. parahaemolyticus* were much higher than that of the control group at 24 h after injection. The ALP (Alkaline Phosphatase) activities of abalone challenged with *V. parahaemolyticus* were significantly higher than those of the control group at 8 h and increased further up to 48 h after the challenge. In contrast, the activities of ALP and ACP in the *E. coli*-challenged group showed no statistically significant differences at any of the sampling times. The activities of SOD (Superoxide Dismutase) in cell free hemolymph from the *V. parahaemolyticus*-exposed group were significantly lower than those of the control group at both 1 h and 24 h, whereas there was no difference in SOD activity observed in the group exposed to *E. coli* at any of the sampling times. The activities of lysozyme and phenoloxidase in *Haliotis diversicolor* were relatively low in both control and bacteria-exposed groups when compared with reports for other invertebrates; no significant difference was found between the infected groups and the control for these two parameters, due to the low activities and high individual variance.

KEY WORDS: *Haliotis diversicolor supertexta*, superoxide dismutase, innate immune, acid phosphatase, alkaline phosphatase

INTRODUCTION

Having an open circulation system, abalone has considerable opportunity to encounter the pathogens and pollutants present in its surrounding water. Although little is known about the innate defense system(s) of abalone, some information is available from the study of other molluscs. To cope with the foreign invaders, many bivalves possess cellular and extra cellular defense mechanisms that are remarkably effective. Hemocytes are the main cellular effectors of invertebrate immunity and are capable of recognizing pathogens in a lectin-mediated process (Pipe 1990, Renwratz 1983) and in phagocytosing, thus isolating and inactivating them (for a review see: Canesi et al. 2002). In addition, cell-free hemolymph of certain bivalves has been shown to contain various biologically active substances involved in innate defense, including agglutinins (e.g., lectins), lysosomal enzymes (e.g., acid phosphatase, lysozyme), toxic oxygen intermediates, and various antimicrobial peptides (Canesi et al. 2002).

In abalone, some research has been focused on the cellular defense system. Lebel et al. (1996) have established that two types of hemocytes are present in the blood of the abalone *Haliotis tuberculata*. Moreover, it has also been shown that salinity stress and certain pollutants can affect the migratory and phagocytic activities of these hemocytes (Chen 1996, Martello et al. 2000). Recently, Shelagh et al. (2003) have shown a direct link between stress and the immune reaction in the abalone *H. tuberculata* using immune parameters such as the number of circulating hemocytes, the migratory activity, the phagocytic capacity, and the respiratory burst responses of the hemocytes.

H. diversicolor supertexta is one of the most commercially important cultured abalone in southern China. However, although

progress in abalone culture has occurred in recent years, abalone culture remains an expensive undertaking, due in part to the catastrophic effects of frequent infectious disease outbreaks (Zhang et al. 2001). To understand the mechanism of innate immunity by which *H. diversicolor supertexta* defends itself against pathogens, we infected *H. diversicolor supertexta* with a nonpathogenic bacterium (*Escherichia coli*) as well as a pathogenic bacterium (*Vibrio parahaemolyticus*) and compared several immunity-related parameters at different exposure times.

MATERIALS AND METHODS

Animals, Immune Challenge, and Hemolymph Collection

Abalone (*Haliotis diversicolor supertexta*) were collected from a commercial farm (Futian, Dongshan, Fujian Province) and maintained in polyethylene tanks, each containing 20 animals in 50 L of aerated and sand-filtered seawater at 23°C to 25°C. Animals were left undisturbed for 2 wk to acclimate to their environment. Abalone were challenged by injecting either 50 L of *Vibrio parahaemolyticus* (isolated from diseased abalone, Zhang et al. 2001) in 0.9% NaCl (6.7×10^7 cells/mL), 50 L of *E. coli* in 0.9% NaCl (6.7×10^7 cells/mL) or 50 L of 0.9% NaCl (as control) into their pleopod muscle. After injection, the abalone were returned to their original tanks containing seawater at the same temperature. Hemolymph (approximately 1.0 mL/animal) from unchallenged or challenged abalone was collected at 1, 4, 8, 12, 24, 48, 96 or 192 h post challenge using a capillary to withdraw fluid from a center incision in the pleopod muscle. Samples were collected into a 1.5 mL centrifugal tube and immediately centrifuged at 150g for 5 min at 4°C to remove hemocytes. Cell-free hemolymph (CFH) samples were frozen at -80°C until used.

Biochemical Assays

ACP and ALP activity assays were carried out according to King's method (Cui 1981) using a commercial kit (Nanjing

*Corresponding author. E-mail: zhangziping@hotmail.com

Jiancheng Bioengineer research institute, China). Analysis was performed in 96-well flat-bottomed microtitre plates and the concentrations of products were monitored spectrophotometrically at 550 nm after incubation of the plate at 37°C for 30 min in the case of ACP, or for 20 min at 37°C for ALP. The activity of ACP and ALP was defined as the amount of phenol (mol) produced under the mentioned conditions per mg protein.

SOD activity assay, a modification of the xanthine-xanthine oxidase assay (McCord & Fridovich 1969) was adapted by using a kit (Nanjing Jiancheng Bioengineer Research Institute, China). The assay was carried out in 96-well flat-bottomed microtitre plates and 1 nitrite unit (NU) of SOD was defined as the quantity of enzyme (per mg protein) that inhibits the reduction of cytochrome c by 50%.

Phenoloxidase activity was measured according to the methods of Horowitz (1952) and Ashida (1971) with L-Dopa used as the substrate. Briefly, 20 µL of cell-free hemolymph samples were added to the wells of a 96-well flat-bottomed microtitre plate in triplicate. To each well, 200 µL of PBS (0.1 mol/L, pH = 6) and 20 µL of L-Dopa solution (0.01 mol/L), were added and the absorbance was read continuously at 490 nm for 15 min. One unit (PO activity) was defined as absorbance change in 490 nm of 0.001 in 1 min and the concentration was expressed as units of activity per mg of protein contained in the sample.

Lysozyme activity was analyzed according to Wilson and Ratcliffe (2000). Briefly, purified lysozyme (100,000 units/mg; Sigma) was diluted in PBS pH 6.2 to give the standards of 0, 2, 4, 8, 12, 16, 20, and 30 g/mL. Aliquots (10 L) of each standard, or the CFH samples were added to the wells of a 96-well flat-bottomed microtitre plate in triplicate. To each well, 250 L of a suspension of lyophilized *Micrococcus luteus* in PBS (0.3 mg/mL; Sigma) was added and the absorbance was continuously monitored at 450 nm for 10 min. For the calculation of lysozyme-like activity, the values for the standards were plotted on a graph of $DA_{450\text{ nm}}/10\text{ min}$ against lysozyme activity (1 unit-change in $A_{450\text{ nm}}$ of 1.0 in 1 min). The values for lysozyme-like activity for each cell-free hemolymph sample were extrapolated from this graph and the concentration was expressed as units of activity per mg protein contained in the sample.

Total protein content of each cell-free hemolymph sample was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

Statistical Analyses

All data are presented as means and standard errors of at least 20 specimens. For comparison of two means, paired or unpaired Student *t*-tests were used where appropriate. $P < 0.05$ was considered as the lower limit of significance, whereas $P < 0.01$ was considered as the most significant difference.

RESULTS

Time Course of ALP and ACP Activities in Cell Free Hemolymph Following Bacterial Challenge

The activities of ACP from abalone injected with *V. parahaemolyticus* were significantly higher than that of control group at 24 h after injection (Fig. 1). In contrast, the activities of ACP between *E. coli* group and control group demonstrated no signifi-

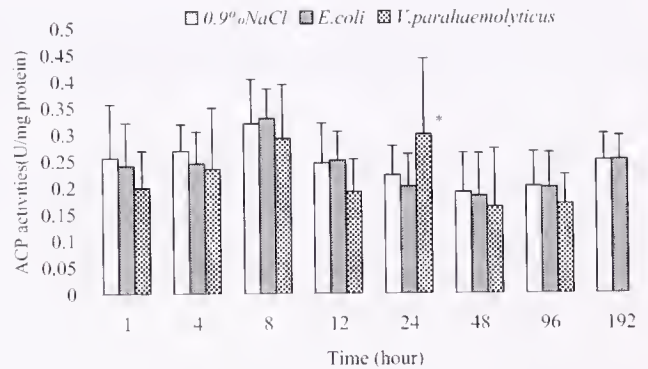


Figure 1. Time course of ACP activities in cell free hemolymph following bacterial challenge. All data are presented as mean and standard errors of a minimum of 20 abalone. * = significant ($P < 0.05$) differences from values of control group (0.9% NaCl injection). All abalone had died at 192 h after injection and no data were available at that time.

cant difference at each sampling time (Fig. 1). All abalone died at 192 h after injection and no data were available at that time.

The ALP activities of abalone injected with *V. parahaemolyticus* were significantly higher than that of the control group at 8 h ($P < 0.05$) and were even more significant at 48 h ($P < 0.01$). Interestingly, at 96 h post exposure the ALP activities of the *V. parahaemolyticus*-challenged group were notably lower than that of control and *E. coli* group (Fig. 2). Same as ACP, the activities of ALP between *E. coli* group and control group demonstrated no significant difference at each sampling time (Fig. 2).

Time Course of SOD Activities in Cell Free Hemolymph Following Bacterial Challenge

The activities of SOD in cell free hemolymph from *V. parahaemolyticus* group were significantly lower than that of control group at 1 h and 24 h. However, there was no statistical difference between the *E. coli*-challenged group and control group at any

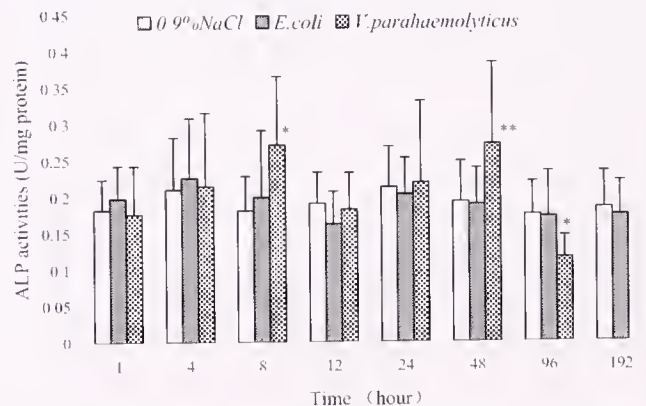


Figure 2. Time course of ALP activities in cell free hemolymph following bacterial challenge. All data are presented as mean and standard errors of a minimum of 20 abalone. * = significant ($P < 0.05$) differences from values of control group (0.9% NaCl injection), ** = the most significant ($P < 0.01$) differences. All abalone had died at 192 h after injection and no data were available at that time.

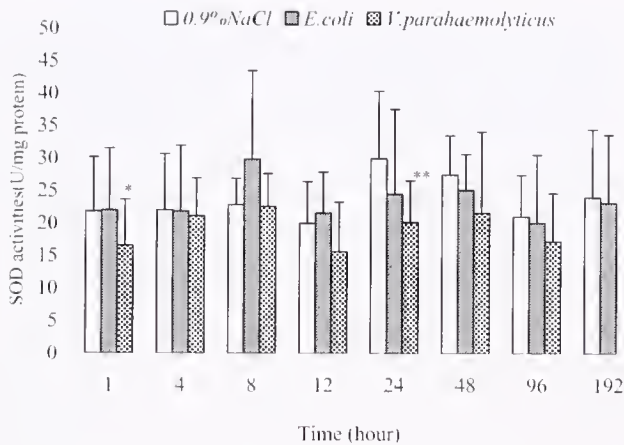


Figure 3. Time course of SOD activity in cell-free hemolymph following bacterial challenge. All data are presented as mean and standard errors of a minimum of 20 abalone. * = significant ($P < 0.05$) differences from values of control group (0.9% NaCl injection), ** = the most significant ($P < 0.01$) differences. All abalone had died at 192 h after injection and no data were available at that time.

sampling time when the activities of SOD were considered (Fig. 3). Moreover, as a general trend, the average of the SOD activity from the *V. parahaemolyticus* group was lower than that of control and *E. coli* group at each sampling time.

Time Course of Lysozyme Activities in Cell Free Hemolymph Following Bacterial Challenge

No significant differences in the activities of lysozyme were observed under any of the exposure conditions and at any of the times of exposure. The degree of variation, in the cell-free hemolymph samples between individual abalones was, however, quite large (e.g., 24 h after injection with 0.9% NaCl, the samples ranged from 0.015–0.472 U/mg) and any underlying trend may have been masked by this extreme variability (Fig. 4).

Time Course of Phenoloxidase Activities in Cell Free Hemolymph Following Bacterial Challenge

The activities of phenoloxidase in *Haliotis diversicolor supertexta* were low (Fig. 5), considerably lower than that reported in

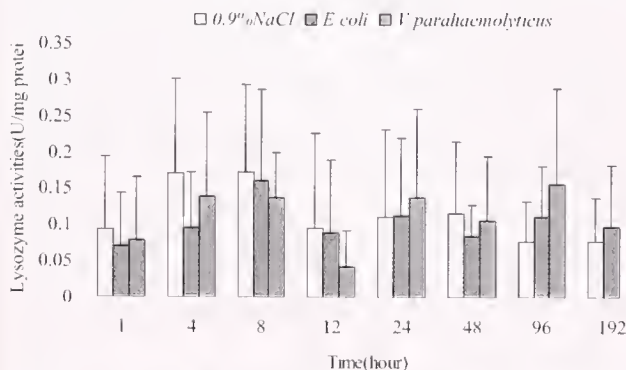


Figure 4. Time course of lysozyme activities in cell free hemolymph following bacterial challenge. All data are presented as mean and standard errors of a minimum of 20 abalones. All abalone in *V. parahaemolyticus* group died at 192 h after injection and no data were available at that time.

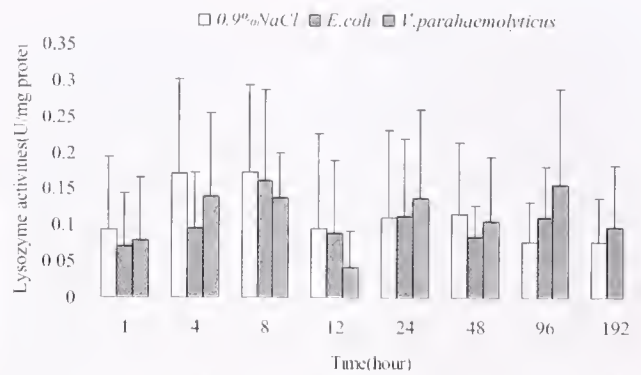


Figure 5. Time course of phenoloxidase activities in abalone following bacterial challenge. All data are presented as mean and standard errors of a minimum of 20 abalone. All abalone had died at 192 h after injection and no data were available at that time.

other invertebrates. For example, values of 2.41–3.46 have been reported in scallop *Chlamys farreri* (Sun & Li 1999). No significant differences were observed under any of the exposure conditions and at any of the times of exposure.

DISCUSSION

A series of immune defense reactions will normally be elicited if bacteria or other pathogenic microbes enter the body of an invertebrate (Cheng 1978). Because invertebrates do not have the capacity to mount humoral and adaptive immune responses, hemocytes play an important part in the shellfish defense system because of their ability to phagocyte, encapsulate, and kill microbes (Zhou & Pan 1997). In addition, processes such as agglutinins, lysosomal enzymes, and toxic reactive oxygen intermediates form during a respiratory burst and may also play an essential role in innate immune responses to clean invading pathogens from the shellfish tissue and hemolymph (Aswanik et al. 1999, Mitta & Vandenbulcke 1999, 2000).

ACP is an important component of the phagocytic lysosome, and is released during the process of phagocytosis and encapsulation (Zhai et al. 1998). Mollusc ACP is found mainly in granulocytes and is responsible for lysis and eventual decomposition of foreign agents including many bacteria. It has previously been shown that the concentrations of lysosome enzymes in some shellfish were increased after they were infected by bacteria (Cheng 1979). In the present study, the activities of ACP and ALP in *V. parahaemolyticus* group were significantly increased at the time points before 24 h and 48 h of the exposure respectively.

These results indicated that *V. parahaemolyticus* induced the ACP and ALP activities and activated the immune defense response in abalone, *Haliotis diversicolor supertexta*. However, the *E. coli* had no effects on ACP and ALP activity in our study, because we observed no significant differences to results obtained from the control group. Our results are in contrast to those reported by Sun and Li (1999) who observed that *E. coli* can also significantly induce ACP and ALP activities in the scallop, *Chlamys farreri*.

It has been previously suggested by Feng (1988) that the elevation of lysosome enzyme (including lysozyme, ACP and ALP)

activity was not only one mechanism of immune defense, but that its depletion may also be a symptom of disease. In the present study, ALP activities declined noticeably at 96 h and ACP activity was substantially decreased at 48 h post injection when the abalone were exposed to *V. parahaemolyticus*. Thus, it may be suggested that the capability of the immune defense in abalone can be disturbed by this pathogenic bacterium and may further be an indicator of the severity of infection in this animal. It was noteworthy that we found some evidence of pathologic changes in abalone injected with *V. parahaemolyticus* (data not shown). Symptoms of the diseased abalone included increased mucus, decreased activity, and loss of absorbability. Furthermore, no samples were available at 192 h after injection due to the high mortality in the *V. parahaemolyticus* injected group (no mortality was observed in either the control or *E. coli* exposed group).

Superoxide dismutase is capable of eliminating superoxide free radicals and may prove protective in bivalves, freeing them from the impact of reactive oxygen species (Nasci et al. 2002). In general, SOD will be induced when the amount of free radical increased. The activities of SOD in cell free hemolymph from the *V. parahaemolyticus*-challenged group were significantly lower than that of the control group at 1 h and 24 h in the present study. Our results are in agreement with the previously reported results of Ding et al. (1996), who infected *Haliotis discus hanna* with *E. coli* or *Vibrio* spp. and found that the SOD activity in cell-free hemolymph was markedly depleted. These phenomena are so called negative-induced, which may also be an indication of immune defense activation in abalone. The reducing of SOD activities allows the relative long existence of reactive oxygen intermediates and is helpful against bacteria. Again, as was observed for the ALP and ACP responses, there was no significant difference between the *E. coli*-treated group and the control when SOD in cell-free hemolymph was considered. These results may indicate that abalone immune defense shows different responses to patho-

genic and nopathogenic bacteria, although the mechanism by which they might be capable of distinguishing such differences remains unclear (*V. parahaemolyticus* is a conditional pathogenic bacterium, it caused abalone disease under our experiment condition. *E. coli*, in general, is a natural bacterium and had no effects on abalone in our study).

The activities of lysozyme, and phenoloxidase in *Haliotis diversicolor supertexta* were low. For example, lysozyme activity of $4. \pm 0.6$ units/mg protein was recorded in cell-free hemolymph of *Blaberus discoidalis* (Wilson & Ratcliffe 2000). While in abalone, *Haliotis diversicolor supertexta*, the mean value was 0.2 units/mg protein below. As mentioned in Results, the activities of phenoloxidase in *Haliotis diversicolor supertexta* were 10 times lower than in the scallop *Chlamys farreri* (Sun & Li 1999). Therefore, it may be that these two parameters may play a less important role in immune defense in abalone. High variability was observed between individual samples.

Further work is required to ascertain the role of these enzymes in abalone immune defense. Fortunately our research group has cloned the SOD gene in abalone, *Haliotis diversicolor supertexta*, and further research to determine why and how the SOD activity is diminished, following challenge by such pathogenic bacteria, is ongoing. Genomic scale investigation is also being carried out in our laboratory on the molecular mechanism of abalone response to pathogenic and non-pathogenic bacteria by using a combination of cDNA microarray and other technology.

ACKNOWLEDGMENT

This work was supported by National High Technology Research and Development program of China (863 program) project (2002AA629220) of China, Natural Science Foundation of Fujian Province project (B0440003) and Department of Education of Fujian Province project (JA004241).

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THE ECOLOGY OF POLYCHAETES THAT INFEST ABALONE SHELLS IN VICTORIA, AUSTRALIA

H. MCDIARMID,¹ R. DAY,^{1*} AND R. WILSON²

¹Zoology Department, University of Melbourne, Victoria, 3052, Australia; ²Museum of Victoria, Carlton Gardens, Victoria, 3051, Australia

ABSTRACT Very little is understood regarding polychaete borers and the effect they have on their hosts. In Victoria and elsewhere in Australia many stunted populations of *Haliotis rubra* have extensively bored shells. This study investigated the infestation by polychaetes of abalone shells and their effect on the health and growth of abalone. Polychaete numbers were estimated by providing diatom food so that they emerged. Borers were most prevalent in the spire region and near closed respiratory pores. In larger *H. rubra* shells, the frequency of polychaete borers increased and borers were present in more parts of the shell. Experiments showed that uninfested *H. rubra* and *H. laevigata* shells from a hatchery could be colonized by *Polydora woodwicki* and *Dipolydora armata* from infested abalone over short periods in the laboratory. Live specimens of *P. woodwicki* were observed for the first time. Larger shells of *H. rubra* were found to be more susceptible to polydorid infestation than large *H. laevigata* or small *H. rubra*, apparently because they provide more suitable sites for settling larvae. Larvae from both species colonized specific places on abalone shells. X-ray photographs were used to determine burrow shapes of *Boccardiella* MoV 3840, *Polydora woodwicki*, and *Dipolydora armata*, which have not been previously described. *Boccardiella* MoV 3840 was found to cause the host abalone to form mud blisters. Rates of burrow growth could be determined using sequential x-rays. High levels of boring were correlated with shell thickness and had a detrimental effect on several condition indices of host abalone, showing that borers have marked detrimental effects on the health and growth of abalone. Thus borers may severely affect fishery yield and productivity.

KEY WORDS: parasite-host interaction, host susceptibility, shell parasites, polydoridae, burrow morphology, burrow growth, abalone productivity

INTRODUCTION

Blacklip abalone *Haliotis rubra* (Leach) are the most abundant abalone species in Victoria and are the basis for a lucrative fishing industry, worth over \$58 million a year in Victoria in 1999 to 2000 (ABARE 2001). This species is also important to the rapidly developing Victorian abalone aquaculture industry. *H. rubra* are known to host a variety of organisms that bore through their shells (Shepherd & Breen 1992). Greenlip abalone (*H. laevigata*, Donovan), which support important commercial fisheries elsewhere in Australia, appear to be less often affected. The major species responsible for this boring belong to the family Spionidae (Annelida: Polychaeta) (Blake & Evans 1973). These polychaete borers, known as polydorids, may have important consequences for the biology of the hosts, and thus for the economics of the fishery and mariculture. At this stage however, there is little hard data to assess these claims.

Most research on shell borers is either taxonomically based or ecologically based. Taxonomic studies often concentrate on one species of boring organism and regularly do not record the host species or the effect on the host. Ecologic studies frequently seem to provide inaccurate descriptions and records of the species of boring organisms present (e.g., Smyth 1990). In either case, information is lost.

Polydorids produce eggs in capsules attached to the burrow, often in strings, and the larvae usually hatch at the 3 setiger stage and develop in the plankton before settlement, but some species are lecithotrophic (Blake & Woodwick 1975, Woodwick 1977, Day & Blake 1979, Sato-Okoshi et al. 1990). The settlement and burrowing behavior of polydorids is poorly understood considering their prevalence in easily studied tidal and subtidal environments and their significance to commercial fisheries (Blake 1996). Blake (p. 85) stated "we know little about how species initially

become established in such a habitat (mollusc shells), the mechanism by which they expand their burrow, how they feed and how they interact with their hosts."

The impact of borers on commercially important bivalves depends on the site of colonization of the host shell (Blake & Kudachnov 1978). Preferences for mollusc hosts and the effects of host size or encrusting algae on colonization by borers are also not currently understood. To understand the relationship between polydorids and abalone, one needs to determine how they become established in a shell, and the factors affecting establishment.

Burrowing by polydorids was initially believed to be carried out mechanically by the chaetae, in particular the modified chaetae of the fifth chaetiger (Blake & Evans 1973). The most recent evidence however, suggests chemical breakdown of the shell instead of a mechanical process (Haigler 1969, Zottoli & Carriker 1974, Sato-Okoshi 1997). The burrows are not often seen to penetrate the inner surface of their host's shell and do not have any direct contact with the host animal, but this seems to be because the burrowing activity induces the host to secrete a protective layer of dark conchiolin, followed by a nacreous shell layer, on the inside of the shell (Haigler 1969, Kent 1979, Blake 1996, Marshall & Day 2001). High levels of burrowing cause some molluscs, including abalone, to significantly increase the shell thickness (Marshall & Day 2001). This repair response, of the mollusk, to holes penetrating the shell takes considerable time to complete (Thomas & Day 1995) and has been suggested to affect the health of the animal and even slow its growth (Kent 1979, Kojima & Imajima 1982, Handley & Berquist 1997, Handley 1998).

The burrows end at two exterior apertures from which the worm feeds (Blake 1996). The boring activities of polydorids result in simple U-shaped burrows, Y-shaped burrows, pear-shaped, complex branching burrows, shallow depressions, or mud blisters (Blake & Evans 1973, Blake 1996). Burrow shape and size is also believed to be a factor in the effect of the borers on the host mollusc (Blake & Evans 1973, Zottoli & Carriker 1974). The rate

*Corresponding author. E-mail: r.day@unimelb.edu.au

of boring of different species may also determine the effect on the host mollusc (Zottoli & Carriker 1974).

There is little known about infestation of abalone (*Haliotis* spp.) by polydorids. A preliminary study (McDiarmid & Wilson, unpublished data) has identified several polydorid species in abalone that do not match any species known for Australia. This, and their presence only near major shipping ports, suggests some may be introduced as "exotic" species. Further taxonomic work is required to establish whether this is the case. The effect on the abalone is believed to increase with the degree of infestation (Blake & Evans 1973, Kojima & Imajima 1982). Abalone with shells weakened by many burrows would provide easier prey for large predators such as fish, stingrays, and octopus (Shepherd & Breen 1992).

This study examines the host-parasite relationship between boring polydorids and *H. rubra* and *H. laevigata*, in particular the effect of infestation on *H. rubra*. New methods are described that can be used to examine colonization behavior and the growth and morphology of the burrows formed and to measure the effect on the host.

MATERIALS AND METHODS

Extraction and Identification

Polychaetes were extracted from abalone shells by placing them in a 50% alcohol, 50% seawater mix. This method caused the worms to either escape their burrows completely and then die where they could be easily collected, or at least expose themselves out of their burrows where they could be extracted manually. An interactive key was constructed (Wilson & McDiarmid 2004), and used in conjunction with a review of the spionids present in southern Australia by Blake & Kudenov (1978) to identify the key features of these taxa.

Pattern of Infestation of Abalone Shells

Seventy abalone of varying sizes collected from Point Cook reef were used to determine whether the area of the shell and/or the level of encrusting algal cover were correlated with the number of polydorids present in the shell. The number of polydorids was determined by placing abalone shells in fresh seawater, to which dead diatoms were added to encourage the feeding behavior of the worms, and observing them under a stereo microscope. Polydorids are easily observed even to the naked eye when they are feeding, because their palps are distinctive (Fig. 1).

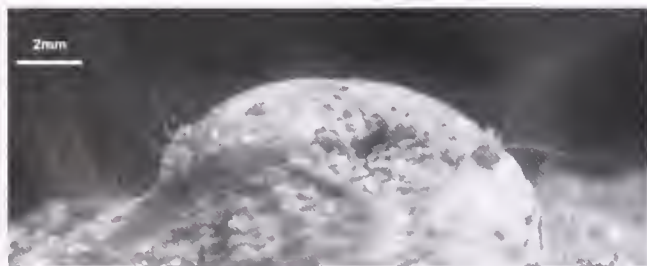


Figure 1. Photograph of the spire of a shell of a blacklip abalone (*Haliotis rubra*), showing two pairs of feeding palps of polydorid polychaetes (*Polydora woodwicksi*) protruding from burrows that extend as mucus tubes above the shell surface. The worms were induced to protrude by mixing dead diatoms into the water.

To determine the pattern of infestation over the shell, shells were divided into three sections: the spire, the area between the ostia and columella and the flat section of the body whorl between the spire and the growing margin (Fig. 2). Each section was observed for a period of 5 min. Counts on every fifth abalone were repeated and the standard deviation of counts was found to be very low ($SD = 2.77$). Algal cover was graded using a scale devised by Smyth (1989). Areas of shells and sections of shells were determined by wrapping the shells in graph paper and counting squares.

The polychaetes were extracted from 15 juvenile (30–60 mm) and 15 adult (>80 mm length) *H. rubra* and identified, to determine any host preferences of the polydorid species.

Colonization of Abalone Shells by Polydorids

Haliotis rubra infested with borers were collected from a depth of 3–4 m off the south eastern boundary of the Point Cook Marine Park in Port Phillip Bay, or at "Fred's Wall," 50 m offshore from the breakwater at Williamstown beach. Several shells from each sample of abalone were set aside and the worms were extracted for identification. One hundred and eighty juveniles of *H. rubra* and *H. laevigata* Donovan, free from borers, were purchased from an abalone mariculture facility for the colonization experiment. All abalone were wrapped in damp towels and placed in cool boxes during transport and left for 1 wk in the aquarium system to acclimatize before experiments. Injured or dying animals were eliminated.

To investigate the colonization behavior of borers and the susceptibility of abalone species to colonization by polydorid larvae, "recipient" hatchery abalone without borers were placed in contact with infested wild abalone from Point Cook reef ("source" abalone) carrying a known number of polychaetes. Recipient abalone were all checked to make sure no boring polydorids were present. They were then placed in either direct or indirect contact with infested abalone, using tanks either containing infested abalone or receiving water from a tank containing an infested source abalone. This experiment was conducted in tanks provided with flowing

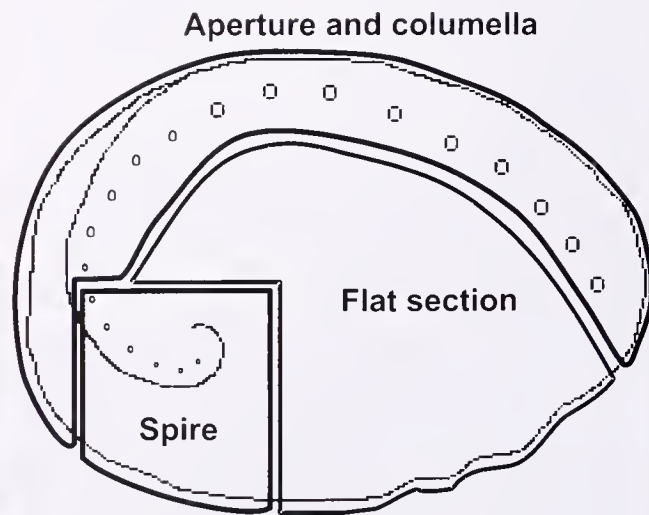


Figure 2. Diagram of an abalone shell, indicating the shell areas used to count polydorids on 70 abalone shells, to determine patterns of infestation in different sized abalone. These areas are: the spire; the area between the ostia and the columella; and the flat section of the body whorl of the shell.

seawater from a large cooled recirculating seawater system, with temperature ranging from 11 °C to 15 °C.

Eighteen abalone with 30 to 40 polydorids present in the shell were used as source abalone in the "direct" colonization treatments. Three abalone that had ~180–220 polydorids were selected to be used as source abalone in the "indirect" colonization treatments. Several shells from the same sample were set aside and the polychaetes were extracted for identification.

In each of the 9 direct colonization replicates for *H. rubra* and *H. laevisgata*, two (30–35 mm) juvenile and one (60–65 mm) subadult abalone were used as recipients and were placed in 20-L polypropylene buckets along with an infested abalone (Fig. 3). In the indirect colonization experiment, water from a tank with the heavily infested abalone was led into 6 jars (6 L) containing 3 recipient abalone (one ~60 mm and two ~30 mm). Three jars contained *H. rubra* recipients, and three contained *H. laevisgata*. There were three blocks of this arrangement (Fig. 4). Nine 20-L buckets containing *H. rubra* and nine containing *H. laevisgata* were used as controls in a similar set up to the direct colonization treatment, without infested abalone. The replicates for each treatment and control were randomly placed, and assigned to alternating water supply outlets to reduce effects due to water flow rate.

The experiment was carried out in constant darkness, because this is believed to induce reproduction of the polychaetes (Evans 1969) and also seems to reduce stress on the abalone (previous observations). The tanks were cleaned every 4 days and fed artificial abalone food every 2 days. If an infested source abalone died it was replaced with another one carrying a similar number of polydorids. After 72 days shells from each of the treatments were closely examined under a dissecting microscope and the number of polydorids and the locations of their burrows were recorded. The polydorids were then removed for identification.

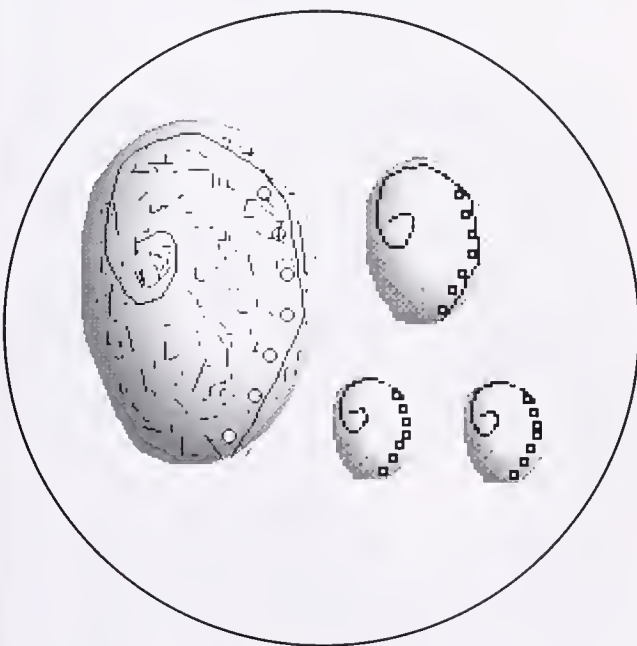


Figure 3. Diagram showing the arrangement of abalone in each of 9 replicates of the "direct" colonization experiment for both *Haliotis rubra* and *H. laevisgata*. One large infested abalone (~30 worms) was placed alongside one medium sized (~60mm) and two small (~30mm) uninfested abalone. In controls the infested abalone was omitted.

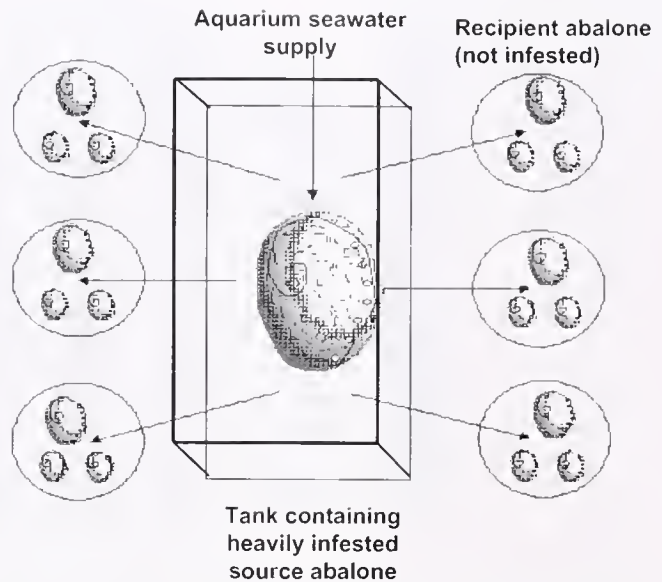


Figure 4. Diagram showing the arrangement of abalone in each of the 3 blocks of the "indirect" colonization experiment. As indicated by arrows, water flowed from the tank with an infested abalone into 6 jars containing uninfested recipient abalone of the same sizes as in Figure 3. Three jars contained *Haliotis rubra* and 3 contained *H. laevisgata*.

Burrow Morphology and Growth Rate

To determine the morphology and rate of expansion of the burrows, abalone shells with live polychaete borers from the wild and from the colonization experiment were x-rayed at the end of the colonization experiment and again 29 days later. Both adult and juvenile polychaete borers were investigated. X-rays were taken using a Hewlett Packard Faxitron x-ray system. An exposure of 30 Kv, 0.2 mA for 12 sec was used. After the last x-ray was taken the polychaetes were removed from the shell to relate the burrow morphology to the species producing it. Burrow areas were determined by tracing them onto graph paper, and the percentage increase between x-rays in the overall size of the burrow was calculated.

Effects on Host Abalone

To relate the condition of abalone to the abundance of borers in their shell, 65 *H. rubra* from Williamstown of a size range 95–110 mm were haphazardly selected. Each abalone's length and width was measured and the percentage of the shell bored was estimated using a grading system adapted from Handley (1997, 1998) (Table 1). Repetitive tests were carried out on 20 of the shells to determine the precision of this method. Eighty percent of these tests

TABLE 1.

The definitions of the grades used to describe bored abalone shells.

Grade	Percentage of Shell Bored
1	0% to 10%
2	10% to 30%
3	30% to 60%
4	60% to 80%
5	80% to 100%

produced the same grade and the remaining ones were within one grade of the original.

The abalone body was removed from the shell and placed on absorbent paper to remove excess water, to determine the body wet weight. Wet muscle weight was recorded after the head and viscera were removed. Dry muscle weight was obtained by placing it in a drying oven at 60°C for 48 h or until a constant weight was achieved (Davenport & Chen 1987, Roper et al. 1991, Handley 1998).

The thickness of the shell was measured using modified vernier calipers at the top of the spire. Abalone are known to thicken the shell in response to borers (Marshall & Day 2001) and the extra shell deposition presumably diverts resources from other tissues (Blake & Evans 1973, Shepherd & Breen 1992). Because the abalone were collected just after the spawning season, the effect of boring on reproductive condition could not be determined.

Three indices were used to measure the condition of the abalone:

- Wet weight of muscle divided by the wet animal weight (CI_{wet}). Healthy animals would present a higher ratio because the muscle tissue is used to store glycogen as an energy reserve (Carefoot et al. 1993). Note that the gonads of all abalone were spent.
- Dry muscle weight divided by wet muscle weight (CI_{dry}). This index determines the amount of water in the muscle. Healthier muscles have a lower water content (Carefoot et al. 1993, Handley 1998).
- The width divided by the length of the shell (CI_{wl}). Faster growing abalone (and thus presumably healthier) are known to lengthen the shell more in comparison to the shell width (Oakes & Fields 1996, Worthington et al. 1995).

RESULTS

Pattern of Infestation of Abalone Shells

There was a significant correlation between the shell area and the number of polychaete borers in the shell ($r = 0.698$, $P < 0.001$), but a linear regression explained only about half of the variation in the data ($R^2 = 0.478$) (Fig. 5). The deviations of the data from the line suggest that shells below about 1800 mm² (22 mm length) are seldom infested, and that the number of borers may increase more rapidly with size in larger shells. When the

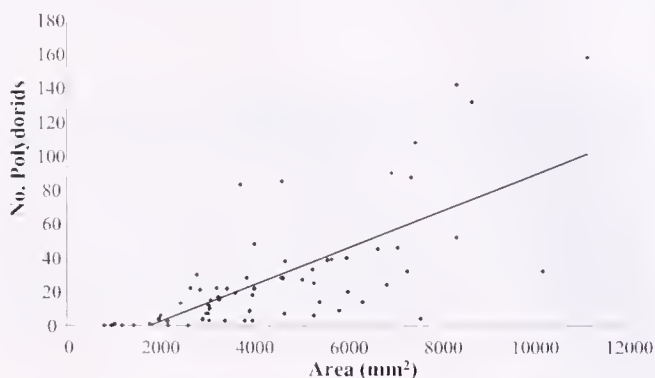


Figure 5. Scatterplot of the number of polydorids present in 70 abalone shells against shell area. The correlation coefficient was 0.698, but the fitted linear regression shown explains only about half (0.449) of the variation in polydorid numbers.

residuals for the number of borers versus shell area were plotted against the grades of encrusting algal cover, there was no obvious relationship. This may be because encrusting algal cover was correlated with shell area ($r = 0.746$).

Table 2 shows the species of polychaete borers present on different sized abalone. Only 4 species were found in abalone <60 mm length, and *Dodecacaria* sp., which was present in 83% of large shells, was absent in these smaller shells. Only the spire area was infested (usually in the groove between the whorls) in smaller shells (30–50 mm), while in progressively larger abalone increasing proportions were infested around the closed ostia and the columella (Fig. 6). Only abalone >100 mm were commonly infested in the flat section of the body whorl. Many borers in the flat section were observed near spirorbid or other serpulid polychaete tubes attached to the shell surface.

Colonization of Abalone Shells by Polydorids

None of the abalone in controls and none of the smaller juveniles (30–35 mm) in other treatments became infested. The presence/absence of infesting polydorids in the subadult abalone (60–65 mm) was analyzed (Table 3). Note that the use of these data avoids the assumption that each polydorid larva colonized the abalone independently.

H. rubra was found to be significantly more susceptible to infestation by borers than *H. laevigata* ($\chi^2 = 4.96$, $df = 2$, $P < 0.05$). The infested *H. rubra* were also each colonized by more polychaetes than infested *H. laevigata*, so that the total number of polychaetes present in all shells was greater in *H. rubra* (Fig. 7). The proportion of *H. rubra* and *H. laevigata* infested with polydorids was not significantly different in the “direct” and “indirect” treatments ($\chi^2 = 1.56$, $df = 1$, $P = 0.3$, combined test for both hosts). This result should be regarded with caution because the numbers of shells infested are small in this experiment, but it is

TABLE 2.

Species of boring polychaetes present in the shells of juvenile and adult abalone at Williamstown. Numbers are percentages of shells in which each species was found. A blank indicates the species was not found. Museum of Victoria numbers refer to their cataloguing system. Descriptions of taxa are available from Robin Wilson at the Museum of Victoria.

Borer Species	Juvenile Abalone (30–60 mm) (n = 15)	Adult Abalone (>80 mm) (n = 15)
<i>Boccardia chilensis</i> (Blake and Woodwick)		16.7
<i>B. MoV 3833</i>		16.7
<i>Dipolydora armata</i>	33.3	58.3
<i>D. MoV 3834</i>		8.3
<i>D. MoV 3835</i>		8.3
<i>D. MoV 3836</i>		8.3
<i>D. MoV 3838</i>	6.6	8.3
<i>Dodecacaria</i> sp.		83.3
<i>Polydora giardi</i> (Mesnil)		16.7
<i>P. MoV 3842</i>	6.6	16.7
<i>P. woodwicki</i>	33.3	33.3
<i>Pseudopolydora</i> MoV 3837		16.7
Total species present	4	12

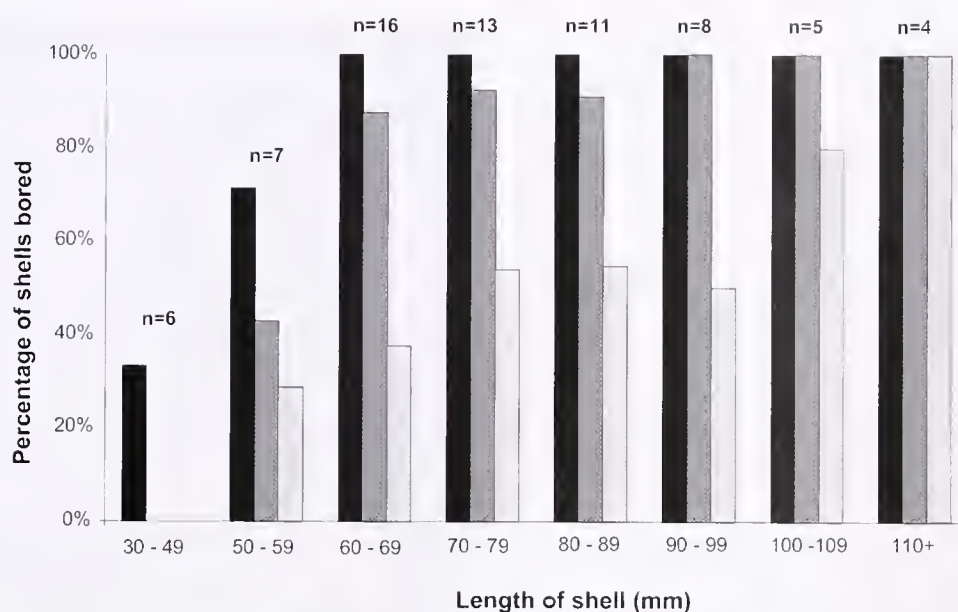


Figure 6. The percentage of 70 abalone of different sizes that were infested with borers in particular areas of the shell. Black bars: spire area. Dark grey bars: area from ostia to columella. Light grey bars: flat area of body whorl. Sample sizes are shown at the top of each bar.

clear that polydorid larvae can be carried in the water to infest new hosts.

The number of borers in each section of the shell expected by chance, based on the relative area of each section, was calculated. For each abalone species the observed numbers differed significantly from those expected (*H. rubra*: $\chi^2 = 150.4$, $df = 3$, $P < 0.001$, *H. laevigata*: $\chi^2 = 26.2$, $df = 3$, $P < 0.001$). The pattern of infestation did not differ significantly between the two abalone species ($\chi^2 = 4.259$, $df = 4$, $P > 0.05$) (Fig. 8). These results should be viewed with caution however, due to the low numbers for *H. laevigata* and the fact that this analysis assumes each spionid larva colonized independently. In summary *H. rubra* are more susceptible than *H. laevigata* and borers are more common than expected in the spire, closed ostia and below the small calcareous tubes of polychaetes attached on top of the abalone shells.

Two species of spionid polychaetes, *Polydora woodwicki* (Woodwick) and *Dipolydora armata* (Langerhans), infested the abalone in this experiment. Several larvae of polydorids were observed crawling over the surface of juvenile abalone shells until they found a suitable crevice. These larvae were then observed to begin burrowing at these sites, but subsequent observations after a short period of time often showed they were no longer present, leaving the burrow half started. Those that did stay and produce large burrows were found to be *P. woodwicki*. *Dipolydora armata*

burrows were much smaller and larvae of this species were not observed.

Burrow Morphology and Growth Rate

During this experiment it was observed that the majority of polychaetes present in a shell survived the death of the abalone. Their survival depended on the shell staying upright and being placed in an area of water flow and available food. The ability of the polychaetes to survive in an empty shell for a period of time allowed X-rays to be taken. Further, they did not bore through the inner surface of the shell during the 46 days that the dead shells were held.

Because several of the worms died after the first x-ray, presumably due to the stress of the transport involved, the expansion

TABLE 3.

The number of shells in which borers were observed in each treatment of each species of abalone (results from 60–65 mm abalone only).

Treatment Recipient Abalone	Direct (n = 9)	Indirect (n = 9)
<i>H. rubra</i> (blacklip)	7	3
<i>H. laevigata</i> (greenlip)	2	3

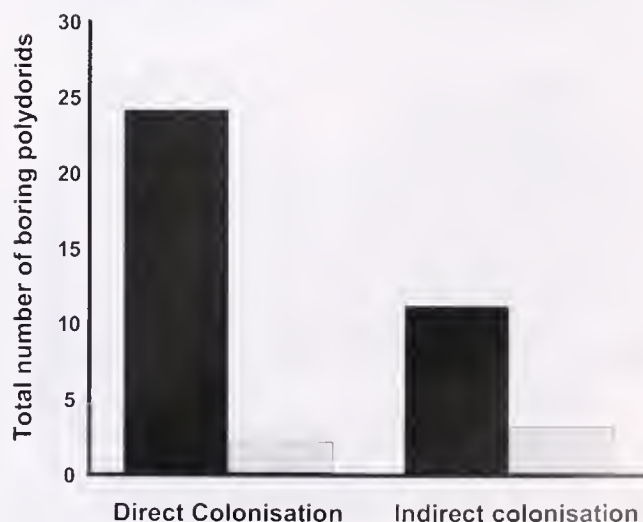


Figure 7. The total number of polydorids found infesting shells of each abalone species in each treatment of the colonization experiment. Black bars: *Haliotis rubra*. Grey bars: *H. laevigata*.



Figure 8. Numbers of polydorids boring in particular locations on shells of each abalone species in the colonization experiment. Black bars: *Haliotis rubra*. Grey bars: *H. laevigata*.

of only 3 burrows was observed over the 29 days between x-rays (Fig. 9). Two of the burrows were formed by *Polydora woodwicki* and increased their sizes by 18.9% and 27.6% respectively, the third burrow was formed by *Dipolydora armata* and increased its size by 15.9% over the 29 days.

The shape of the burrow can be seen clearly in several of the x-rays; and burrows from the two species were found to be very distinctive. *Dipolydora armata* exhibited extensive and intricate burrows, whereas *P. woodwicki* had a distinct U-shaped burrow. The U-shape of burrows for *Boccardiella* MoV 3840 (sp. nov.) are also clearly visible in Figure 10A, and over the entire abalone shell in Figure 10B. This species was found only in abalone from Mallacoota. Note that these burrows do not interconnect, and change direction just before crossing another burrow. They are distinctive and clearly different to burrows of *P. woodwicki*. A mud blister, in

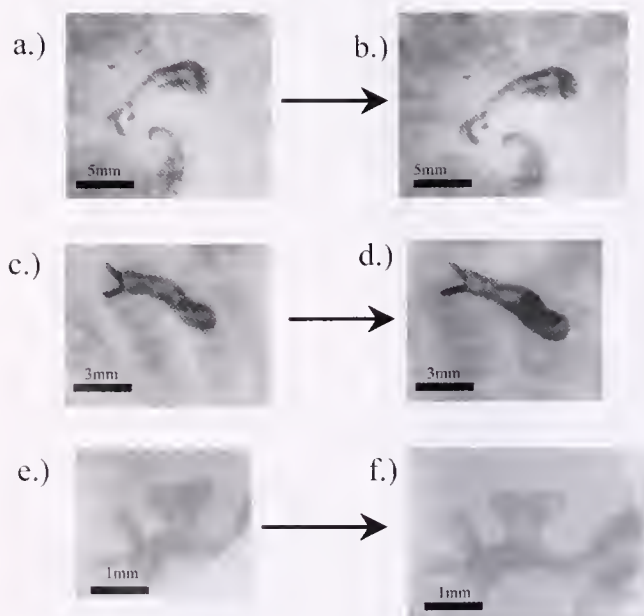


Figure 9. Expansion of polydorid burrows. a to d. Size of burrows formed by *P. woodwicki* at the end of the colonization experiment (72 days after start) and 29 days later. a to b. The burrow increased in size by 18.9%. c to d. Burrow size increased by 15.7%. e to f. Burrow formed by *D. armata* at end of colonization experiment (72 days) and 29 days later. Burrow size increased by 27.6%.

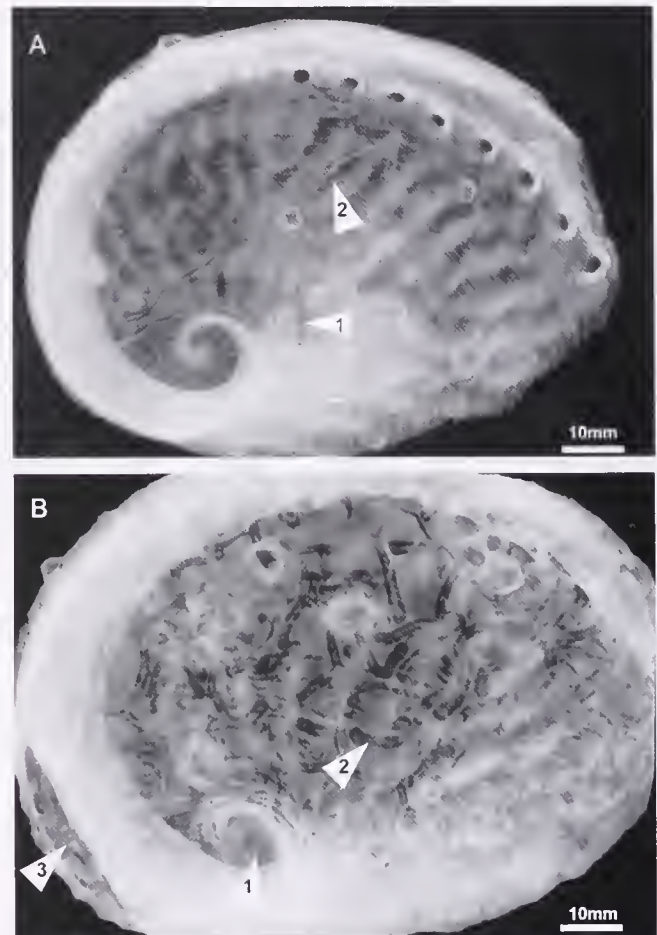


Figure 10. X-rays of abalone shells from Mallacoota showing the difference between heavily bored and lightly bored shells. A. Lightly bored abalone shell, with a few burrows of *Boccardiella* MoV 3840 (1). Tubes on the shell surface formed by serpulid worm are also visible (2). B. Heavily infested abalone shell with burrows through the entire shell, formed by *Dipolydora armata* (1), and by *Boccardiella* MoV 3840 (2). A mud blister formed by *Boccardiella* MoV 3840 beneath the columella area of the shell is shown at (3).

which the abalone has walled off a space inside the shell with a new shell layer, is also shown in Figure 10B, and in cross-section in Figure 11. All blisters were found to be full of sediment and *Boccardiella* MoV 3840. Thus, X-rays (Figs. 9, 10B) can be used to identify burrows made by *P. woodwicki* and *Boccardiella* MoV 3840 and the smaller intricate burrows formed by *Dipolydora armata* and also reveal mud blisters, as well as the extent to which borers have colonized a shell.

Effects on Host Abalone

Many heavily bored shells were brittle and were shattered during the process of removing the abalone soft tissues, so that shell parameters could not be recorded. A Pearson correlation matrix of all the indices measured showed they were not correlated with each other or the spire thickness, so that they represent independent measures of condition. Both wet muscle weight as a proportion of body wet weight and the ratio of dry to wet muscle weight decreased significantly as the extent of boring increased ($F = 16.604$; $df = 1, 58$; $P < 0.001$ and $F = 7.655$; $df = 1, 58$; $P =$

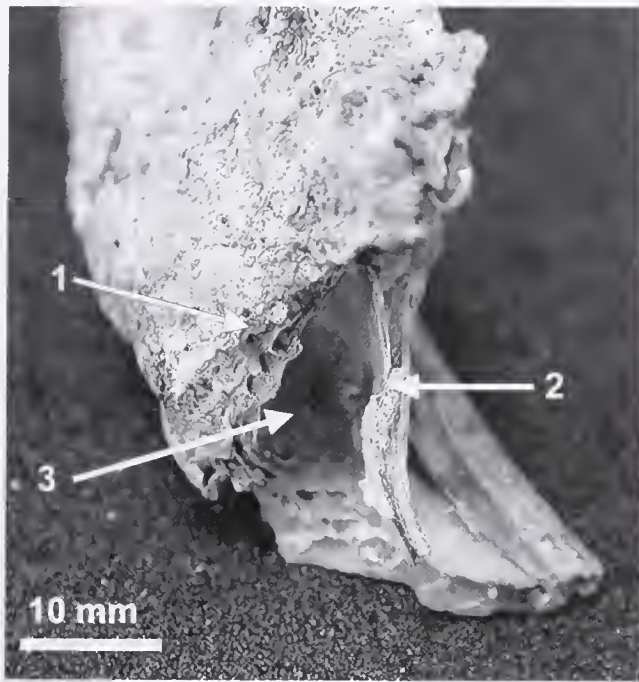


Figure 11. The edge and columella of a blacklip abalone shell from Mallacoota. There is evidence of heavy boring in the outer shell (1). The abalone has formed a mud blister by laying an extra layer of shell inside the normal shell surface (2). The compartment (3) formed by the abalone is filled with sediment and polydorids. The polydorid responsible does not match any currently described species from Australia (*Boccardiella* MoV 3840).

0.008 respectively). The width to length ratio increased significantly with increased boring ($F = 4.638$; $df = 1, 58$; $P = 0.035$). We did not find good evidence that the thickness of the shell at the spire increased with more extensive boring ($F = 1.402$; $df = 1, 31$; $P = 0.245$), perhaps because spire thickness would relate specifically to boring beneath the spire. Boring changed shell shape in other ways (Fig. 12). The inner surface is greatly deformed, and the overall shape of the shell is also affected in heavily bored abalone: it is wider than the lightly bored shell and the columella shelf is enlarged.

DISCUSSION

Pattern of Infestation of Abalone Shells

The positive relationship between the size of the shell and the number of borers found in this study has also been observed in the abalone *Haliotis diversicolor* Reeve (Kojima & Imajima 1982), and in other molluscs (Mohammad 1972). We also found that the diversity of borers increased with host size. Both relationships would follow from the longer exposure to borers of older shells, and the fact that a larger shell area would provide a larger target for planktonic larvae to encounter and allow more borers to burrow. Adult abalone do not hide in crevices as juvenile abalone do, and thus would experience more water flow. They may also encounter more settling borers. Shell structure may also influence the degree of boring, as adult abalone shells are eroded, particularly at the spire. It is possible that the outer layer of prismatic calcite is more resistant to borers (Thomas & Day 1995), so that boring increases once it has worn away on the older spire area. Note that the closed

ostia are plugged with aragonite nacre, and this is another area where borers are common.

The larger number of species in larger shells may also be due to some being secondary borers. *Dodecaceria* sp., which was present only in large shells, requires previous boring to be present in the shell (Gibson 1978). Such secondary borers recolonize unused burrows and extend these burrows for their own use (Smyth 1990).

Species common on smaller shells are likely to be primary colonizers. Identifying the primary borers is important, because preventing boring by these species would prevent the establishment of other borers. The colonization experiment reported here definitively identifies *Dipolydora armata* and *Polydora woodwicki* as primary colonizers, and as having planktonic larvae. Both were common on small abalone. Several species found on juvenile shells were not much more common in adult shells. These species may be suppressed by other borers, or the epibiota on larger shells.

Colonization in the Laboratory

Polydora woodwicki and *Dipolydora armata* were found to easily reproduce in tanks, and thus may affect aquaculture farm productivity and economic performance. Their planktonic larvae appear to settle in short periods, and could spread easily in mariculture facilities.

The laboratory experiments were designed to examine colonization while eliminating confounding influences in field observations. Our observations of the larval behavior of *P. woodwicki*, and the fact that *Haliotis rubra* were more susceptible than *H. laevigata* to colonization, suggests that small crevices associated with shell sculpture facilitate colonization by this polydorid. Shells of *H. rubra* are more sculptured than those of *H. laevigata*, and the spire and ostia are much more pronounced. Further, few borers were found in the flat parts of the shell, and these were often beside the tubes of spirorbids on the shell. Nothing was known about the colonization behavior of *P. woodwicki* prior to this study, but Zottoli & Carriker (1974) found that *Polydora websteri* (Hartman) preferred shell crevices on the oyster *Crassostrea virginica* and the mussel *Mytilus edulis* Lamarck. Blake (1996) suggested crevices provided the juvenile with a place to form a simple tube, to anchor itself and then initiate a burrow.

The observations of *P. woodwicki* larvae colonizing are the first observations of this type for this species. Borers that left burrows

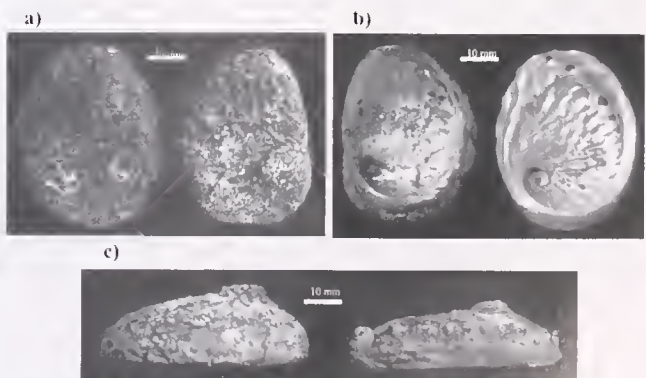


Figure 12. Effect of extensive boring on the size and shapes of abalone shells. a. Top view. b. Bottom view with animal removed. c. Side view. Extensively bored shell on the right in (a) and on the left in (b) and (c).

unfinished may have either died or left the burrow deliberately. The larvae may begin to burrow at one site on the surface of the shell and then decide that the site is unsuitable after they begin burrowing. The fact that the majority of the full sized burrows formed in juvenile shells were in the thickest sections of a shell, and that burrows did not connect, suggests borers can detect the shell thickness. Note that over a period of two and half months the worms did not bore through the inner surface of empty shells. Previous authors have suggested that polydorids do not break the inner surface of the shell because the abalone constantly produces extra layers of shell (Marshall & Day 2001). Thicker shell would actually allow the borers to increase the size of the burrows.

Size has an effect on both the number of boring worms present in the field, and on colonization, as only abalone 60 mm in length or larger became infested during this experiment. This suggests that larvae find it difficult to settle and bore into smaller shells. Kojima & Imajima (1982) found that the smallest *H. diversicolor* infested was 29 mm in length, but the majority of the boring occurred in abalone larger than 45 mm. Size also has a significant effect on the number of borers present in other mollusc shells (Smyth 1990). Perhaps this is to do with the smoothness of juvenile shells and the lack of crevices. Smaller shells are also very thin, making it difficult for a borer to form a burrow.

As reported previously by Leonart & Handlinger (1998), polydorids continue to live in the shell after the host has died, as long as the shell stays in a position that allows them to continue filter feeding. These worms will hasten the degradation of the shell, and thus play a role in the longevity of dead mollusc shells. This should also be considered by ecologists who measure mortality using empty shells, or study their use by hermit crabs (Smyth 1990).

Burrow Morphology and Growth Rate

The U-shaped burrows of *Boccardiella* MoV 3840 have not previously been observed. This seems to be an undescribed species. The shape of the burrow for *Dipolydora armata* concurs with those observed in several other substrates (Blake & Kudenov 1978) but it has not previously been observed in abalone. The U-shaped burrow for *Polydora woodwicki* has not previously been observed, and specimens of this species have been collected only once before.

We have shown that x-rays are useful to measure burrow expansion rates, although only a few borers could be kept alive for subsequent x-rays. Methods to improve survival during handling are needed, to facilitate further work to determine burrow expansion rates of other species.

The size and number of burrows formed in 72 days during the colonization experiment demonstrate how quickly polydorids can become established in, and damage shells. The X-rays show the extent of the damage well. This method will allow measurements of rates of infestation and will be an important tool to estimate burrow morphology and rate of expansion in different borer species and thus to determine which species cause greatest damage to the host. Extraction of borers from heavily infested shells does not pinpoint which species inflicted the damage.

Effects on Host Abalone

This study shows that boring affects the condition of abalone. Both condition indices, reflecting storage reserves in the muscle

and the health of the muscle, declined significantly with increased boring damage. The increased relative width of shells with more boring, and the other shape changes, indicates that the way heavily bored abalone enlarge their shells is different to those abalone with little boring present. The change in shell shape is obvious when lightly and heavily infested shells are compared. This change may be due to the disturbance caused by the deposition of extra shell layers (Marshall & Day 2001). Heavy boring at specific locations in the shell, such as the growing edge, may also cause the abalone to change the way it grows. The marketability of abalone as a live product and their shells as ornaments and jewelry is severely reduced by high levels of boring.

The mud blisters observed in abalone from Mallacoota have been observed in other molluscs, particularly oysters (Blake & Evans 1973), and can greatly reduce the internal volume of the shell. Thus a larger shell would be required to house the same soft tissue volume of the abalone. The species that caused these blisters also forms large burrows over the entire shell. It does not match any current descriptions from Australia and further work is urgently needed to determine if this species is an introduced or exotic species.

If the correlations reflect causation, then declines in condition indices with increased boring mean that boring affects the health and growth of abalone, and thus the muscle weight recovered by the fishery will be reduced by borers. Similar correlations have been found for other abalone and molluscs in general. *Haliotis diversicolor* had a reduced flesh weight when 10 or more polychaetes were present boring in the shell (Kojima & Imajima 1982). Other work on mussels and oysters has shown that heavy infestations by borers were associated with lower condition indices or reduced nutrient reserves (Kent 1979, Wargo & Ford 1993). In contrast, Clavier (1989) found no correlation between level of boring in *H. tuberculata* Linnaeus and the health of the abalone, but his study did not include heavily bored abalone, where the effect on muscle weight was found to be strongest in this study. Caceres-Martinez et al. (1998) found no significant effect of boring on condition indices of the oyster *Crassostrea gigas* (Thunberg), but again, their study did not include heavily infested oysters. They noted this was essential in determining the relationship between host and borer.

One would expect deleterious effects of borers on host condition because the shell thickening response would shift resources away from other functions. Bored *H. rubra* were found to increase shell secretion rates 4-fold (Marshall & Day 2001). Wilbur & Saleuddin (1983) suggested that one quarter to one third of the total energy of growth is required for shell deposition in molluscs. Thus substantial energy would be required for the increased shell thickening, with a corresponding decrease in resources for somatic growth or fecundity.

The correlation of borer infestation with an increased shell width to length ratio suggests reduced growth. Slow growth in *H. rubra* is indicated by increased width and height relative to length (Worthington et al. 1995). Abalone divers classify stocks in some areas as "stunted," because few abalone grow past the size limit (Wells & Mulvey 1995, Troynikov et al. 1998). Stunted stocks have both high, domed shells, and high levels of boring. Slowly growing abalone may reach older ages because they are not harvested, and thus accumulate many borers. Thus slow growth may lead to heavy boring, or the deleterious effects of boring may cause the stunted growth. Perhaps both effects occur. Further investiga-

tion of juveniles in stunted areas is needed to understand the link between boring and stunted growth.

Abalone extensively infested with sabellid polychaetes were reported to be less able to right themselves when turned over (Oakes & Fields 1996), apparently due to the doming of the shell due to abnormal shell deposition at the growing edge but perhaps also because of the diversion of resources to extra shell deposition. The effects of polydorids on shell shape are less severe.

Decreased growth, decreased muscle weights, and deformation of the shell would all reduce the profit of the commercial abalone industry directly. Polydorid borers may cause such effects. Reduced growth reduces fecundity, and boring weakens the shell, which would increase mortality. This in turn would lower the levels of fishing populations can sustain. If, as we suggest, borers

in abalone cause these effects, they deserve attention. Yet there has been so little research that even the risk of new introduced species affecting fishing areas cannot be assessed. This study provides methods for future work to study colonization, burrow morphology and expansion, and the effects on abalone hosts.

ACKNOWLEDGMENTS

The authors thank the Museum of Victoria and the Zoology Department and Dentistry Hospital at the University of Melbourne for the facilities provided and Ocean Waves Seafoods Pty Ltd. for the abalone. The authors also thank Prof. J. Clement, Cameron Dixon, and John Ahern for assistance and discussions, and referees for the many important suggestions.

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DATA ON PINK ABALONE, *HALIOTIS CORRUGATA* (GRAY 1828) WITH INFESTED SHELLS FROM THE SAN BENITO ARCHIPELAGO, BAJA CALIFORNIA, MEXICO

F. CABALLERO-ALEGRÍA, J. A. RODRÍGUEZ-VALENCIA,* AND J. CASTRO-GONZÁLEZ

Instituto Nacional de la Pesca, CRIP-Ensenada, Carretera Tijuana-Ensenada Km 107, Parque Industrial Fondeport, El Sauzal, Baja California, Mexico

ABSTRACT This work presents abundance estimations of pink abalone (*Haliotis corrugata*, Gray 1828) with shells infested by epibionts from the San Benito archipelago (Baja California, Mexico). The first quantifications, undertaken at the end of the 80s, revealed that the highest abundances of infested abalone were concentrated around one of the three islands of the archipelago, where notorious differences in aspect and sizes between infested and normal exemplars were observed. Between 1989 and 1992, infested pink abalones from this location were commercially extracted, attempting to clean up the population. Extractions resulted in the dominance of normal exemplars. Nevertheless, 10 y later (year 2001), the abundance of infested pink abalone was again higher than that of normal pink abalone, and normal and infested organisms seemed to be bathymetrically segregated. In year 2001, differences in aspect between infested and normal organisms remained notorious, but differences in size were practically absent. Most of the infested exemplars were adults. Two morphotypes of infested pink abalone were identified: (a) abalone which shells are dome-shaped and which growth is vertically directed, with the surface homogeneously covered by epibionts and (b) abalone with flat and oval-shaped shells, which growth is horizontally directed, and edges usually free of epibionts.

KEY WORDS: abalone, *Haliotis corrugata*, epibionts, shell, infestation, Mexico

INTRODUCTION

In Mexico, all abalone species have high economic value. Nowadays, the production of meat and shell is supported by green *Haliotis fulgens* (Philippi 1854) and pink abalone *H. corrugata* (Gray 1828). Sometimes, shells of green abalone can be as expensive as the meat itself, whereas those of pink abalone usually occupy the second place in importance, because they accumulate high densities of epibionts. The presence of epibionts does not affect the quality of the meat, but some can perforate the shell destroying the mother-of-pearl. This kind of damage is typical for the sponge *Cliona* (Kojima & Imajima 1982), the spionid polychaete *Polydora* (Clavier 1992), and some sabellid polychaetes (Leighton 1998, Kuris & Culver 1999).

The knowledge about the effect of epibionts over Mexican abalone species is scarce. Alvarez-Tinajero et al. (2000) reported about the incidence of the boring clam *Lithophaga* over pink abalone and its repercussions over the shell marketing at Cedros Island. Cáceres-Martínez and Tinoco-Orta (2001) analyzed the effect of spionid and serpulid polychaetes over *H. rufescens*. Nothing is known about the frequency of infested abalone at wild populations. Ortiz-Quintanilla (1974) reported for the first time about a population of pink abalone from the middle island of the San Benito archipelago, where the incidence of infested abalone with sizes below the minimum legal size of capture (140 mm of shell length) was particularly high. Due to the appearance of the infested organisms, the local fishermen named them “dwarf abalones.” Between 1989 and 1992 special permissions were granted for extracting infested abalones from the archipelago, attempting to clean up the population.

Here, we present the most important results of the quantifications of infested pink abalone undertaken between 1988 and 1991 at the San Benito archipelago, which up to now have remained unpublished. Additionally, results of the latest quantification un-

dertaken in July 2001 will allow us to know about the persistence of the infestation phenomenon in this area.

MATERIALS AND METHODS

The most important features of the San Benito archipelago (Fig. 1) were described by Rodríguez-Valencia and Caballero-Alegría (2002). The first quantification of infested pink abalone was undertaken in February 1988, randomly locating sampling points inside the fishing area (0–30 m depth) around the three islands, and using a 1 m²-quadrant as sampling unit. All abalone found inside the quadrant were collected. In this survey, 200 m² were explored around the westerly island, 225 m² at the easterly island, and 450 m² at middle island. After this attempt, quantifications for infested pink abalone were restricted to the middle island (October 1989, March 1990, and October 1991) using the method described by Rodríguez-Valencia and Caballero-Alegría (2002). The most recent survey was done in July 2001, again applying the method described by Rodríguez-Valencia and Caballero-Alegría (2002), at 86 randomly located sampling points (Fig. 1).

RESULTS

The Abundance of Infested Pink Abalone During 1988 to 1991

In February 1988, the abundance of infested pink abalone at the middle island of the archipelago was higher (0.3 abalone*m⁻²) than at the westerly and easterly islands (0.1 and 0.08 abalone*m⁻², respectively). Sixty percent of all the pink abalone captured during this survey was infested, and 96% of them were smaller than the minimum legal size of capture (140 mm of shell length). In October 1989, the abundance of infested pink abalone at the middle island was 0.2 abalone*m⁻², whereas at the other two islands was 0.1 abalone*m⁻². Ninety-eight percent of all the abalone collected around the middle island (36996 exemplars) was infested, and 89% of them were below the minimum legal size of capture. The most common epibionts observed over the shells were *Corallina* spp., hydrozoans, bryozoans, serpulid and spionid polychaetes, *Cliona celata*, and *Lithophaga plumula*. Normal and in-

*Correspondence author. E-mail: alejandro.rodriguez@web.de

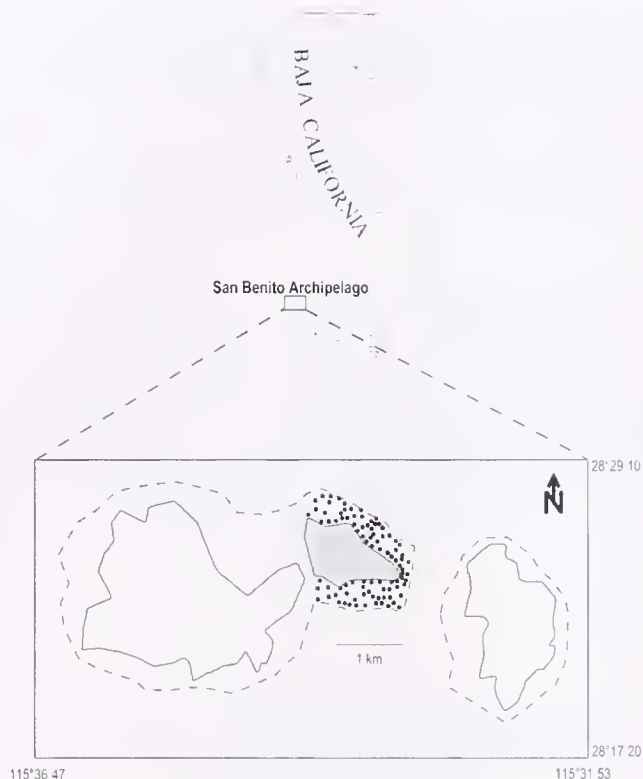


Figure 1. Location of the San Benito archipelago (the middle island is shadowed). Points around the middle island indicate the sampling stations for the survey undertaken in July 2001. The dashed line represents the 30 m isobath.

infested abalone differed markedly in size. In 1989, the modal size of infested organisms was 118 mm versus 140 mm in normal ones. In 1990, modal sizes were 124 mm versus 135 mm, respectively.

As a result of the special capture permissions for infested pink abalone granted to the local fishermen, 6.9, 12.9, 10.0, and 5.8 tones of meat were extracted from the middle island of the archipelago, in 1989, 1990, 1991, and 1992 respectively. In consequence, the abundance of infested pink abalone decreased ($0.2 \text{ abalone} \cdot \text{m}^{-2}$ in March 1990 vs. $0.05 \text{ abalone} \cdot \text{m}^{-2}$ in October 1991), whereas the abundance of normal pink abalone remained between $0.2\text{--}0.3 \text{ abalone} \cdot \text{m}^{-2}$. The contribution of normal pink abalone to the total catch increased from 2% in 1989, to 34% in 1992, whereas that of infested pink abalone below the minimum legal size of capture decreased from 40% to 4% for the same period. The modal shell length of infested pink abalone increased from 127 mm to 142 mm.

The Abundance of Infested Pink Abalone 10 Years Later

In July 2001, the abundance of infested organisms at the middle island was significantly higher than that of normal organisms ($0.1 \text{ abalone} \cdot \text{m}^{-2} \pm 0.03$ vs. $0.07 \text{ abalone} \cdot \text{m}^{-2} \pm 0.03$, respectively; $F_{(1,170)} = 5.0$, $P < 0.05$). Fifty-seven percent of all the pink abalone collected (294 exemplars) was infested. The highest abundance of normal pink abalone was concentrated between 10–20 m deep (52% of their total abundance), whereas 40% of the total abundance of infested pink abalone was found between 20–30 m deep.

Differences in appearance between normal and infested pink abalone were still marked (Fig. 2). In dorsal view, infested shells differ by having only one pair of open respiratory orifices; in ventral view their edges lacked a marked CaCO_3 accumulation band, reflecting a decreased horizontal growth. Nevertheless, differences between the mean shell lengths of normal and infested pink abalone were absent ($127.6 \text{ mm} \pm 1.6$ in infested abalone vs. $129.1 \text{ mm} \pm 2.2$ in normal abalone; $F_{(1,167)} = 1.2$, $P > 0.05$). Significant differences between the mean weight of muscle were also absent ($143.7 \text{ g} \pm 7.0$ for infested abalone vs. $145.6 \text{ g} \pm 9.0$ for normal abalone; $F_{(1,167)} = 0.1$, $P > 0.05$).

In pink abalone, the size of the muscular scar over the ventral face of the shell increases as the organism gets older; therefore, exemplars without or with small scars are younger than those with large and well-defined scars. This allowed us to build groups of relative age (Fig. 3): (1) Juveniles I, as exemplars, which shells lack of muscular scar; (2) Juveniles II, as organisms which shells have small muscular scars; and (3) Adults, as organisms which shells have large and well-defined scars. Four percent of the infested pink abalone was Juveniles I ($120 \text{ mm} \pm 8.0$ of mean shell length), 25% were Juveniles II ($128.5 \text{ mm} \pm 3.0$ of mean shell length), and 73% were Adults ($128.6 \text{ mm} \pm 1.0$ of mean shell length). The mean shell length between these three groups differed significantly ($F_{(2,166)} = 4.1$, $P < 0.05$), but differences were caused by the mean length of Juveniles I (after Tukey HSD Test).

Two morphotypes were recognized among infested abalones (Fig. 4). Organisms with almost perfectly rounded and dome-shaped shells represent the first type. The surface of these shells is homogeneously covered by epibionts. Flatted and oval-shaped shells, without dome appearance, represent the second type. Edges of these shells are usually free of epibionts. Morphotypes were named "vertical growth" and "horizontal growth," respectively. Sixty percent of all the infested pink abalone belonged to "vertical growth" being their abundance concentrated between 10–20 m in depth (55% of their total). The rest of the infested pink abalone (40%) belonged to the "horizontal growth" type, being mainly concentrated between 10–30 m deep (89% of their total).

DISCUSSION

Unfortunately, raw data of the first surveys were not captured and got lost, being only summarized in technical reports of our institution. This hinders the extraction of more information through additional analyses. It is probable that this data set represents the unique documentation about the temporal persistence of the infestation phenomenon by epibionts in abalone, since any other similar information has been published until now. As far as we know, any other attempt to clean up populations of infested organisms, as the one described here, has been done. This strategy, applied until 1992, apparently succeeded because the incidence of infested organisms decreased and the abundance of normal pink abalone remained practically constant. Nevertheless, the absence of the raw data set hinders the statistical confirmation. Ten years later, the abundance of infested abalone again surpassed that of normal exemplars at the middle island of the archipelago, but both showed lower abundance levels than those observed during 1988 to 1991 due to the regional decline in abalone populations (Guzmán-del-Prío 1992; Shepherd et al. 1998). Based on the yearly surveys undertaken at the archipelago (Rodríguez-Valencia

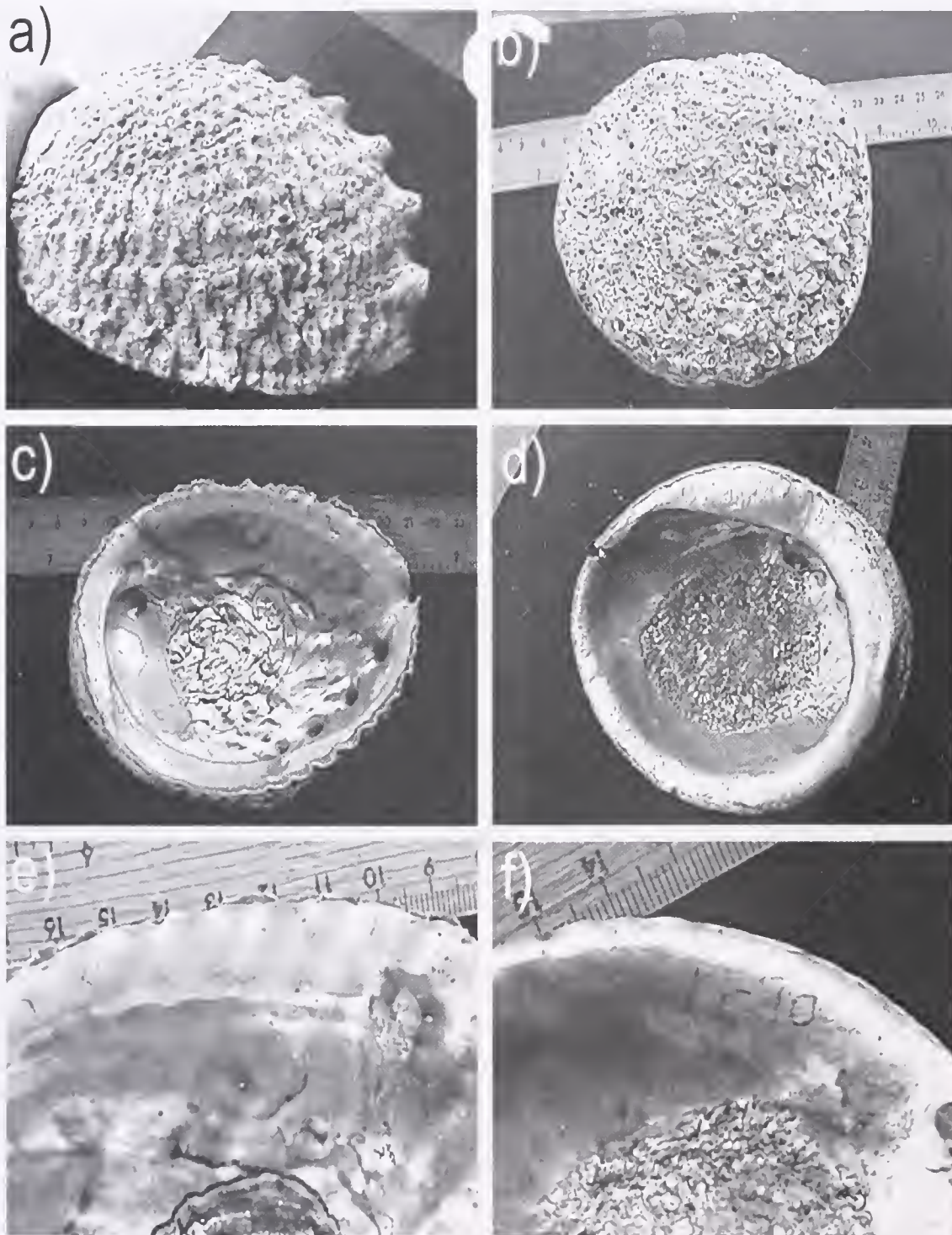


Figure 2. (a) Dorsal view of a shell belonging to a normal pink abalone. (b) Dorsal view of a shell belonging to an infested pink abalone. (c) Ventral view of a shell belonging to a normal pink abalone. (d) Ventral view of a shell belonging to an infested pink abalone. (e) Detail of the ventral edge of a shell belonging to a normal pink abalone. (f) Detail of the ventral edge of a shell belonging to an infested pink abalone.

& Caballero-Alegría 2002), we know that the incidence of infestation by epibionts remains highest around the middle island of the archipelago.

We believe that the abundance turnaround of infested abalone and the absence of differences in size between normal and infested organisms, observed in July 2001, are by-products of the fishery.

This may have occurred because infested pink abalone are not preferred by commercial divers, because their shells will not sell well; therefore, they are focused on the extraction of normal pink abalone. This leads to the reduction of abundance of normal organisms and the reduction of the mean size of normal abalone to sizes similar to those of infested pink abalone. This is supported by

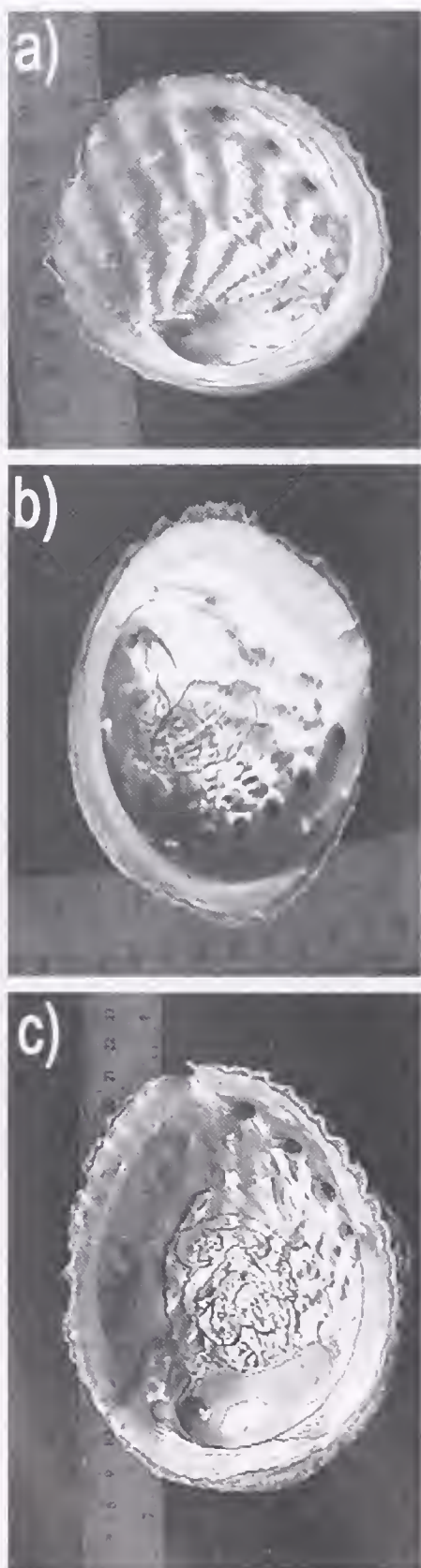


Figure 3. Relative age groups based on the size of the muscular scar, located on the ventral face of pink abalone shells. (a) Juveniles I, without muscular scar. (b) Juveniles II, with small muscular scar. (c) Adults, with large and well-defined muscular scar.

findings of Rodríguez-Valencia and Caballero-Alegria (2002), indicating that between 1989 and 1999 the modal size of pink abalone at the archipelago has decreased.

We still do not have a clear explanation for the occurrence of two morphotypes of infested pink abalone. We hypothesize, that organisms showing "vertical growth" were infested at early ages and small sizes; therefore the epibionts crust covered the whole shell surface and hindered the continuation of horizontal growth. On the other hand, organisms with "horizontal growth" could have been infested when they were older and their shells were already oval-shaped. It is probable that the epibionts crust did not cover the whole surface, allowing the continuation of horizontal expansion. At the present, we are trying to find a method suitable to test this hypothesis.

This topic leads to the following interesting open questions that would be worthy to assess through experimental approaches: What are the reasons for the bathymetric segregations between normal and infested pink abalone, as well as between morphotypes of infested abalone? Are meroplanktonic larvae of some of the epibionts passively or actively retained around the middle island of the archipelago, leading to a higher larval settlement at this location? How comparable are densities and species composition of epibionts over the abalone shells with those over other hard substrata around the middle island (e.g. boulders, shells of other mollusks, and/or crabs exoskeletons)? Are there seasonal/yearly changes in the specific composition of epibionts over the pink abalone shells? Are there physiologic and/or genetic differences between normal and infested pink abalone? Are there chemical differences in shell composition between normal and infested abalone? Is the infestation degree dependent on ontogeny?

During these years we have been willing to find a method to get more accurate age estimations. Nevertheless, as Muñoz-López (1976) reported, deformed spires and damaged periostracs hinder the age determination using growth rings. Our relative age categories are similar to those defined by Muñoz-López (1976), after finding a good correlation between gonadal maturity and development of muscular scars (he used juveniles, lacking of muscular scar; preadults, with muscular scars slightly marked; and adults, with complete and well defined muscular scars). Talmadge (1964) also used similar classifications.

Fortunately, there are no differences in taste of the meat between normal and infested pink abalone. Nevertheless, shell deformations and shell weakness induced by boring epibionts (Oakes & Fields 1996) do have economic repercussions (Alvarez-Tinajero et al. 2000). We believe that the assessment of the remaining open questions and the analysis of temporal dynamics of infesting by epibionts at a fixed location are worthy tasks.

ACKNOWLEDGMENTS

We dedicate this work to the memory of our colleague A. L. Lelevier-Grijalva, who strongly contributed in the conception and development of the first versions of this manuscript. The authors sincerely thank our colleagues from the National Institute for Fisheries (Mexico) that helped during all the surveys. This work has been made possible thanks to the logistic support of the fishermen cooperative S.C.P.P. "Pescadores Nacionales de Abulón". A. Cota-Villavicencio provided valuable suggestions for this manuscript.

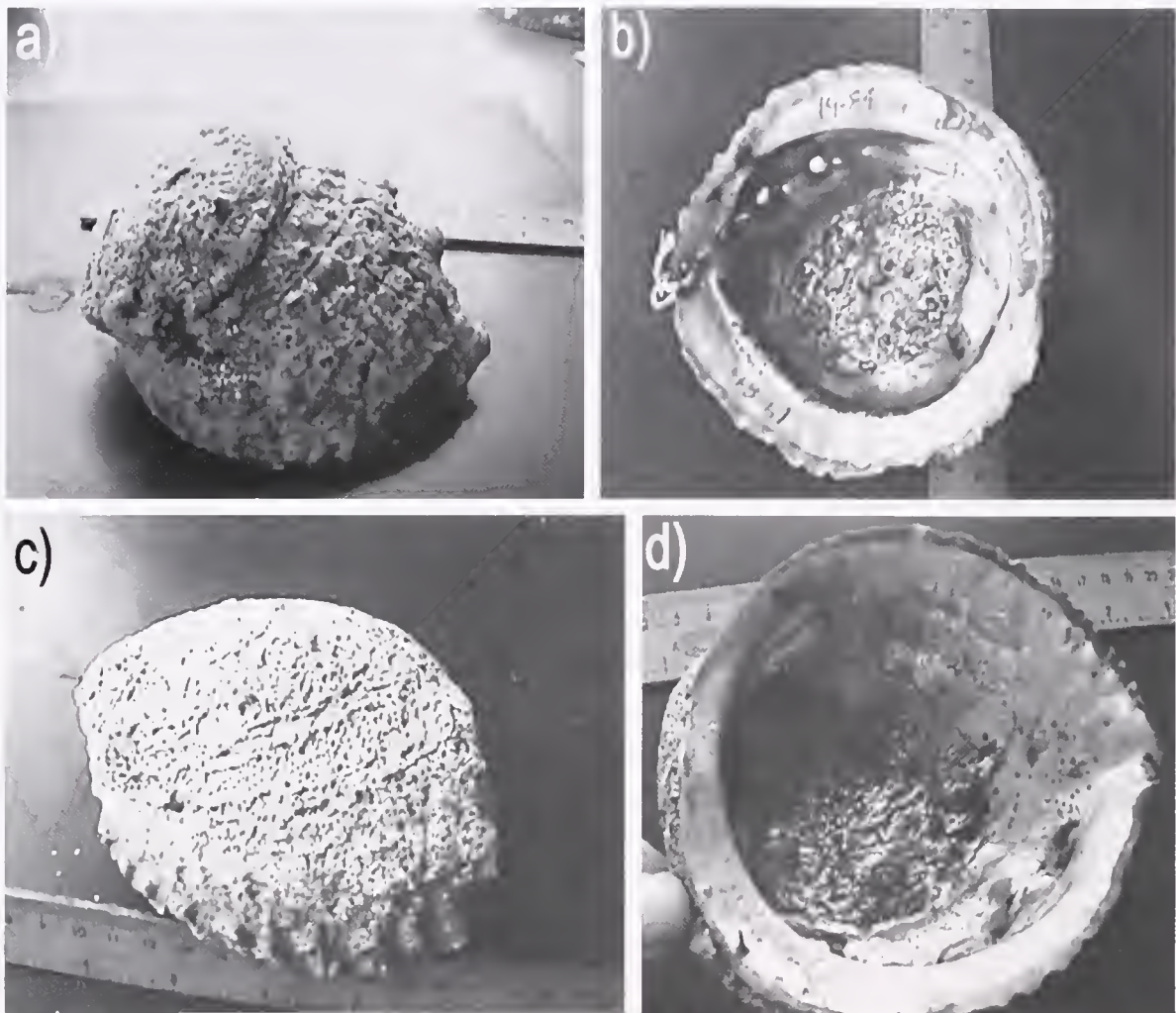


Figure 4. Morphotypes of infested pink abalone from the San Benito archipelago. With "vertical growth": a) dorsal view, b) ventral view. With "horizontal growth": c) Dorsal view, d) Ventral view.

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MICROSATELLITE DNA ANALYSIS OF SOUTHEAST AUSTRALIAN *HALIOTIS LAEVIGATA* (DONOVAN) POPULATIONS — IMPLICATIONS FOR RANCHING IN PORT PHILLIP BAY

BEN T. MAYNARD,¹ PETER J. HANNA,^{1*} AND JOHN A. H. BENZIE²

¹School of Biological and Chemical Sciences, Deakin University, Geelong, VIC 3217, Australia;

²Centre for Marine and Coastal Studies, University of New South Wales, Sydney, NSW 2052, Australia

ABSTRACT The genetic composition of greenlip abalone (*Haliotis laevigata*) from Point Cook in Port Phillip Bay was examined prior to the aggregation of individuals from this site for ranching. The very thinly distributed natural population at Point Cook was believed to be of low genetic diversity, because the animals all originated from a single spawning event 5 y previously. Animals from Point Cook were compared with other *H. laevigata* from two sampling sites within Port Phillip Bay, and two sites outside the Bay in Bass Strait, to examine their genetic diversity and origin. Variation was assessed at five microsatellite loci. Deviations from Hardy-Weinberg equilibrium (HWE) were observed at some loci in various populations, but the Point Cook population was in HWE at all five loci. Mean heterozygosity and number of alleles was similar in all populations. Hierarchical analysis of molecular variance indicated significant genetic variation among populations, but did not differentiate Port Phillip Bay from Bass Strait populations. Pairwise comparisons of multilocus F_{ST} and R_{ST} indicated significant genetic differences between Point Cook and some populations, as well as between other populations, but no consistent spatial pattern of differentiation was observed. There was no significant correlation between genetic and geographic distance. The level of genetic variation observed in the Point Cook individuals was similar to that in individuals from the other four sites, and sufficient to support a ranching program. However, this variation should be monitored to maximize genetic potential, and avoid commercially undesirable effects of inbreeding. Implications of this study in relation to the management of a ranching population in Port Phillip Bay are discussed.

KEY WORDS: microsatellites, genetic composition, *Haliotis laevigata*, ranching population

INTRODUCTION

Australia is a major exporter of abalone, supplying an estimated 81% of fresh and frozen product and 67% of canned product to international markets (Brown 1997), worth over AUD\$249 million (Australian Bureau of Agricultural and Resource Economics 2002). Blacklip abalone, *Haliotis rubra*, is the main commercial catch in the state of Victoria, contributing 7,110 t (worth AUD\$283.8 million) over the past 5 y, during which time greenlip abalone, *H. laevigata*, contributed only 12 t (worth AUD\$474,000) (Anon 2002). The *H. laevigata* fishery is on the verge of closure, and the maintenance of an export industry for this species may only be possible through aquaculture.

Deakin University research, in conjunction with Melbourne Abalone Pty. Ltd., is testing the suitability of ranching abalone in Port Phillip Bay, a large protected Bay relatively isolated from the open sea of the Bass Strait. *H. laevigata* are being accumulated from Point Cook, a northwestern area of Port Phillip Bay, where the dispersed nature of the population suggests they are unlikely to reproduce naturally. Shepherd (1986) found that adult *H. laevigata* aggregate prior to and during spawning, and that aggregation densities of no less than 0.2 animals per m² were essential for successful spawning. Individuals in the Point Cook region are usually more than 100 m apart.

Because the Point Cook population was first observed by divers 4 y ago as spat, it is suspected that these animals are very closely related through a rare spawning event a year prior. It is likely clockwise currents, a predominant pelagic influence in Port Phillip Bay (Black & Mourtikas 1992), could have dispersed larvae to Point Cook from either of the two known *H. laevigata* sources in the Bay at Grassy Point or Queenscliff (Fig. 1). If this were the case, then the Point Cook population could have reduced genetic diversity, because the cohort may have originated from only a few animals. This may give rise to difficulties in a ranching population

derived from these animals, given the higher risk of deleterious inbreeding effects being expressed.

Microsatellites, first described by Weber and May (1989), are short segments of 2–6 base pairs (bp) repeated tandemly in tracts of up to 100 bp long (Chambers & MacAvoy 2000). Microsatellites have been successfully utilized for population genetic research in a range of commercially important and endangered aquatic species, including abalone (Elliott et al. 2000, Huang et al. 2000, Withler et al. 2001, Conod et al. 2002, Evans et al. 2000b, Selvamani et al. 2000, Selvamani et al. 2001, Hanna et al. 2000, Sweijd et al. 2000, Kirby et al. 1998).

Microsatellite primers developed for *H. rubra* have also been successfully utilized with *H. laevigata* (Evans et al. 2001, Robinson & Baranski pers. comm.). We present here the results of a study using five of these microsatellite loci to determine the genetic diversity and possible source(s) of animals being used to develop an artificial population of *H. laevigata* for a ranching trial.

MATERIALS AND METHODS

Abalone Samples

Epipodial tissue samples were collected from live *H. laevigata*, of >100 mm in length, at three Port Phillip Bay sites and two Bass Strait sites during 2002 (Fig. 1). Field sampling was undertaken at Point Cook ($n = 19$) and Grassy Point ($n = 20$), while Queenscliff ($n = 29$), Torquay ($n = 21$) and Phillip Island ($n = 30$) samples were taken at commercial abalone processing facilities. The removal of an epipodium for tissue sampling is considered nondestructive (Withler et al. 2001).

Each epipodium was placed in a 1.5 mL Eppendorf tube containing 70% v/v ethanol and stored on ice during transfer to the laboratory. The ethanol was removed and samples rinsed in 1.0 mL sdH₂O for 5 min to remove exopolysaccharides and trace amounts of ethanol. Excess sdH₂O was then removed and the samples stored at -80°C until analyzed.

*Corresponding author. E-mail: pjh@deakin.edu.au

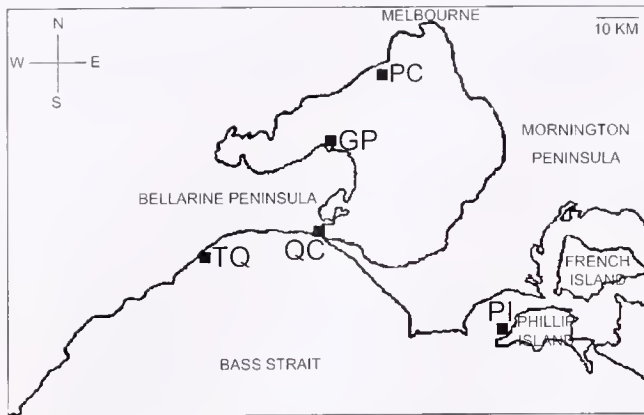


Figure 1. Geographic sites where *H. laevigata* were sampled. PC = Point Cook (thinly distributed, nonreproducing abalone); GP = Grassy Point; QC = Queenscliff; TQ = Torquay; and PI = Phillip Island.

DNA Extraction, Amplification, and Scoring of Microsatellite Loci

Epipodia (~25 mg) were minced with separate sterile scalpel blades and transferred to 1.5-mL Eppendorf tubes, containing 0.5 mL of DNAzol (Invitrogen). Each homogenate was mixed by gentle pipetting, followed by vigorous shaking for 10 min, before centrifugation at 13,500 rpm in a bench centrifuge (Microcentaur) for 10 min at room temperature. Supernatants were transferred to new 1.5-mL tubes and DNA precipitated by the addition of 0.5-mL 100% v/v ethanol per mL of DNAzol. The samples were mixed by inversion, and stored at -20°C for 1 h. To pellet DNA, samples were centrifuged as before. The supernatants were removed and the pellets washed with 1.0 mL 95% v/v ethanol. Each tube was inverted three to six times before centrifugation as before. The ethanol washes were repeated, and the pellets dried at 37°C for 5 min before resuspension in 50 μL of 8 mM NaOH. The resulting DNA solutions were adjusted to pH 7.5 with the addition of 159 μL of 0.1 M HEPES per 1 mL of 8 mM NaOH solution. DNA samples with spectrophotometric purity ratios ($\text{OD}_{260}:\text{OD}_{280}$) between 1.5–2.0 and concentrations of ≥ 20 ng/ μL were stored at -20°C until required.

Any DNA sample with a purity outside of the range 1.5–2.0 was made up to 200 μL with 8 mM NaOH, to which was added 300 μL of sdH_2O and an equal volume (500 μL) of phenol:chloroform:isoamylalcohol (25:24:1). The sample was mixed by inversion before centrifugation as mentioned earlier. The aqueous phase was collected and transferred to a fresh tube. DNA was precipitated from solution by the addition of 2 volumes of 100% v/v ethanol. The sample was stored at -20°C for 30 min before centrifugation as earlier. The DNA pellet was washed twice with 95% v/v ethanol and then dried. The DNA pellet was resuspended in 50 μL of TE buffer.

Five polymorphic loci, viz *RUBGT1* (Huang & Hanna 1998, Genbank Accession Number AF027572), *cmrHr2.14* (Evans et al. 2000a, AF194957), *cmrHr2.23* (Evans et al. 2001, AF302832), *Hrub4*, and *Hrub12* (provided in confidence by N. Robinson and M. Baranski), were amplified from *H. laevigata* genomic DNA.

An FTS-320 Thermal Sequencer (Corbett Research) was used for PCR amplifications, comprising 100 pmol forward and reverse primers (PROLIGO), 1 \times PCR buffer (Invitrogen), 2 mM MgCl_2 (Invitrogen), 2.5 mM each dNTP (Roche), 1 U *Taq* polymerase (Invitrogen), and 200 ng template DNA, made up to 25 μL with

sdH_2O . PCR cycle conditions were 94°C for 2 min; then 35 cycles of 94°C for 15 sec, locus-specific annealing temperature for 30 sec, 72°C for 1 min; and finally 72°C for 10 min. Annealing temperatures were 52°C for locus *cmrHr2.23*, 54°C for locus *cmrHr2.14*, and 55°C for loci *RUBGT1*, *Hrub4*, and *Hrub12*.

PCR samples were mixed with formamide loading buffer (FLB), then denatured at 95°C for 4 min and held on ice. Microsatellite alleles were separated as per Huang et al. (2000), together with three lanes of 10 bp DNA ladder (Invitrogen) in FLB. A nonmutagenic silver staining protocol was used to resolve separated PCR fragments (Ludwig et al. 1989, Caetano-Anolles et al. 1991). Microsatellite DNA sizes were determined as per Huang et al. (2000).

The allele present in the most common homozygote specimen of each locus was sequenced, after TA cloning into pCR2.1 (Invitrogen), to ratify that the product amplified contained the repeat length equal to that being scored after PAGE and silver staining. The correct number of repeats was observed within each sequence, viz sizes 153 bp, 282 bp, 259 bp, 199 bp, and 242 bp for loci *RUBGT1*, *cmrHr2.14*, *cmrHr2.23*, *Hrub4*, and *Hrub12* respectively.

Statistical Analysis

Allelic frequencies were calculated using MS Excel. Arlequin version 2.001 software (Schneider et al. 2000) was used to statistically analyze genotypic data. Observed (H_o) and expected (H_e) heterozygosities were calculated for each locus at each site. Conformity to Hardy-Weinberg Equilibrium (HWE) was analyzed by exact tests using a Markov chain. Multilocus R_{ST} values (assuming the Step-wise Mutation Model for microsatellite evolution) and F_{ST} values (assuming the Infinite Allele Model) were calculated for each population pairing. Probability values for HWE and pairwise tests were Bonferroni-adjusted to account for multiple comparisons (Rice 1989). Mantel tests to analyze correlations between F_{ST} or R_{ST} genetic distances and geographic distance (direct water distance) matrices were also used. Analysis of molecular variance (AMOVA) was used to partition genetic variation into hierarchical levels (among populations, F_{ST} ; among groups of populations, F_{SC} , viz Port Phillip Bay and Bass Strait; and among populations within groups, F_{CT}) to test for spatial structure at different scales.

RESULTS

The number of alleles per population ranged from 2–4 at *cmrHr2.23*, 2–5 at *Hrub4*, 5–8 at *cmrHr2.14*, 7–11 at *RUBGT1*, and 7–14 at *Hrub12* (Table 1). Mean number of alleles ranged from 5.2 at Grassy Point to 8.0 at Queenscliff and Phillip Island (Table 1).

Significant homozygote excesses were observed in 8 out of 25 locus by sample tests, with no obvious association with a particular locus or population (Table 1). Samples from Point Cook were in HWE at all five loci (Table 1). Mean heterozygosity ranged from 0.394 at Grassy Point to 0.596 at Queenscliff (Table 1).

Ungrouped AMOVA comparing all sites across all loci showed significant genetic subdivision among populations ($F_{ST} = 0.011$, $P < 0.001$). However, when populations were grouped into Port Phillip Bay (Point Cook, Grassy Point, Queenscliff) and Bass Strait (Torquay, Phillip Island) sites, there was no significant difference between groups ($F_{CT} = 0.001$, $P = 0.075$), but there were still differences among populations within groups ($F_{SC} = 0.010$, $P < 0.001$).

TABLE 1.

H. laevigata statistics of five microsatellite loci for five sites, including number and size range (bp) of alleles and Hardy-Weinberg results at each site.

	Point Cook	Grassy Point	Queenscliff	Torquay	Phillip Island	Mean
<i>RUBGT1</i>						
No. alleles	7	9	11	8	10	9.0
Allele size	147–165	137–155	137–161	145–161	133–155	
H _o	0.684	0.611*	0.667*	0.760*	0.857	0.716
H _e	0.771	0.835	0.868	0.830	0.825	0.826
n	19	18	24	25	21	21.4
<i>cmrHr2.14</i>						
No. alleles	5	6	8	10	8	7.4
Allele size	278–294	270–290	270–302	266–310	270–302	
H _o	0.538	0.571	0.762	0.700*	0.562*	0.627
H _e	0.757	0.796	0.872	0.842	0.821	0.818
n	13	14	21	20	16	16.8
<i>cmrHr2.23</i>						
No. alleles	2	2	3	4	4	3.0
Allele size	259–265	259–265	259–267	259–269	259–269	
H _o	0.500	0.389	0.385	0.550	0.389	0.442
H _e	0.433	0.475	0.527	0.610	0.600	0.529
n	16	18	26	20	18	19.6
<i>Hrub4</i>						
No. alleles	3	2	4	3	5	3.4
Allele size	199–225	197–199	189–219	195–199	195–203	
H _o	0.091	0.000	0.444	0.100	0.200*	0.167
H _e	0.338	0.454	0.484	0.284	0.618	0.436
n	11	6	9	10	20	11.2
<i>Hrub12</i>						
No. alleles	13	7	14	11	13	11.6
Allele size	220–280	238–260	220–276	228–276	220–270	
H _o	0.917	0.400*	0.722*	0.857	0.933	0.766
H _e	0.931	0.921	0.911	0.910	0.929	0.920
n	12	10	18	14	15	13.8
Mean						
No. alleles	6	5.2	8	7.2	8	
H _o	0.546	0.394	0.596	0.593	0.588	
H _e	0.646	0.696	0.732	0.695	0.758	
n	14.2	13.2	19.6	17.8	18	

H_o, observed heterozygosity; H_e, expected heterozygosity.

* Significant departure from HWE ($P < 0.01$ after Bonferroni correction for multiple tests); n, number of individuals scored.

F_{ST} and R_{ST} values from comparison of population pairs showed statistically significant ($P < 0.001$) differences between some populations (30% to 40% of comparisons), but F_{ST} and R_{ST} values were significant for different populations except for Point Cook × Phillip Island, where both were significant (Table 2). Except for significant differences between Torquay with both Queenscliff and Phillip Island using R_{ST} , all other significant differences were between Point Cook and the other populations (Table 2). No obvious differences in the frequency of dominant alleles, or the presence of rare and unique alleles among populations, were observed to account for pairwise results (data not shown).

Mantel tests of the relationship between genetic distance and direct water distance were not statistically significant for F_{ST} ($r = 0.223$; $P = 0.292$) and for R_{ST} ($r = 0.310$; $P = 0.194$).

DISCUSSION

H. laevigata were rare (less than 1% of the frequency of *H. rubra* at Point Cook; abalone divers W. Butler and B. Cronin, pers. comm.), and were also low in numbers at the other two Bay

sites. Therefore, sample sizes were limited to approximately 20 within Port Phillip Bay. Although below sample sizes used in allozyme assays (e.g., 84 *H. rubra* per population, Brown 1991; 66 *H. laevigata* per population, Brown & Murray 1992), these were

TABLE 2.

Pairwise multilocus F_{ST} (above diagonal) and R_{ST} (below diagonal) values of five microsatellite loci for five sites.

	Point Cook	Grassy Point	Queenscliff	Torquay	Phillip Island
Point Cook	—	0.039*	0.017	0.025*	0.047*
Grassy Point	0.102	—	−0.006	0.001	−0.002
Queenscliff	0.143*	0.062	—	−0.007	0.007
Torquay	−0.018	0.011	0.093*	—	0.002
Phillip Island	0.280*	0.085	0.020	0.126~	—

* Significant value at $P < 0.01$ after Bonferroni correction for multiple tests.

within the range of recent microsatellite analyses on molluscan species that have provided useful data (e.g., 10 *H. rubra* per population, Huang et al. 2000; 10–59 brown mussels per population, Holland 2001).

Small sample sizes are common with species that are endangered, but such small sample sizes may fail to detect relatively common alleles that contribute to significant differentiation among populations (Berg & Berg 2000). Differences in sample size, and in the cohorts sampled, between populations on small sampling scales can bias comparisons of genetic diversity, although this factor would impact allelic variation more than heterozygosity. Further work at our study site will improve discriminatory power by increasing sample size and sampling effort, simultaneously reducing the probability of Type I and II errors (Richardson et al. 1986). More individuals would need to be typed before several unique alleles observed in the Point Cook population, and other alleles uniquely present elsewhere, can be used for stock identification.

Genetic Diversity

Levels of microsatellite polymorphism were similar to previous studies of abalone in southern Australia (Huang et al. 2000, N. Elliot pers. comm.). Levels of allelic variation in the Point Cook population were at the low end of the range for four out of the five loci tested in the present survey. Huang et al. (2000) also found limited genetic diversity in the Point Cook *H. rubra* population, where only two alleles were observed at locus RUBGACA1 compared with four or more in oceanic populations. There were few significant deviations from HWE, concordant with results from previous genetic studies of abalone (Hamm & Burton 2000, Hancock 2000, Conod et al. 2002). The fact that heterozygote deficits were not found at all loci showing significant differences between populations suggests that the Wahlund effect (resulting from the mixing of populations that differ in gene frequency) is not responsible for the deficits observed. Gel images were also carefully checked according to a suite of widely used criteria (B. Huang pers. comm.). Although null alleles have been ruled out for locus RUBGT1 in *H. rubra* (Huang et al. 2000), Conod et al. (2002) concluded that null alleles were the most likely explanation for homozygote excess observations at *cmrHr2.14* and *cmrHr2.23*, and these may explain the deficits heterozygote in *H. laevisgata*.

Spatial Structure

Although there were significant differences between the *H. laevisgata* Point Cook population and other Port Phillip Bay and Bass Strait populations, there was no evidence for a consistent difference between Bay and Strait populations. This information contrasts with published data for *H. rubra*, where the Point Cook population was genetically distinct from oceanic populations in the central Victorian fishery management zone ($\Phi_{PT} = 0.077$ – 0.199 , $P < 0.001$ – 0.027), believed to result from the semi enclosed geography of Port Phillip Bay (Huang et al. 2000).

The level of genetic subdivision in *H. laevisgata* populations found in the present study (overall $F_{ST} = 0.011$, $P < 0.001$) is lower than that recorded for *H. laevisgata* by Huang (1998) in populations throughout Southern and Western Australia using randomly amplified polymorphic DNA ($\Phi_{PT} = 0.184$ over all populations, $\Phi_{RT} = 0.131$ among states, $\Phi_{PR} = 0.107$ among three Western Australian populations), and by Brown and Murray

(1992) and Shepherd and Brown (1993) using allozymes ($F_{ST} = 0.014$). Genetic subdivision in coastal populations of the related species, *H. rubra*, was also greater (overall $F_{ST} = 0.077$) (Huang et al. 2000).

Previous surveys of genetic variation in abalone species in Australia have demonstrated both strong patterns of isolation by distance in *H. rubra*, with average neighborhood sizes estimated to range from 60–70 km (Huang et al. 2000) to 500 km (Brown 1991), and no relationship between geographic and genetic distance among samples from Victoria and Tasmania (Conod et al. 2002). The present study found no significant correlation between genetic and geographic distance. An estimate of neighborhood distance based on comparison of the Point Cook and Phillip Island populations, separated by 100 km, supports data from Huang et al. (2000), and concurs with inferences from ecological studies that abalone are locally recruited (McShane et al. 1988, McShane 1992, Prince et al. 1988).

The present study provided no evidence that Port Phillip Bay populations constitute a group separate from Bass Strait populations (in contrast to *H. rubra* [Huang et al. 2000]), but the Point Cook population was differentiated from all but one other population using F_{ST} (and none of the other populations were differentiated from each other). F_{ST} is influenced to a greater degree than R_{ST} by shifts in allele frequencies, and the greater differentiation with F_{ST} than with R_{ST} supports an interpretation that differences between the Point Cook population and all others is recent and related to a founder effect rather than the accumulation of differences resulting from mutation.

The spawning of abalone is affected by several local environmental factors (Shepherd & Laws 1974, Sasaki & Shepherd 1995). Subsets of animals in close proximity can spawn asynchronously (McShane et al. 1986) or exhibit independent spawning seasons (Shepherd & Laws 1974), providing the opportunities for local selection. Drew et al. (2001) demonstrated that Port Phillip Bay *H. rubra* were less affected than Bass Strait animals by heat and salinity, and differ in physiology (e.g., the inability of oceanic specimens to volume regulate compared with Point Cook abalone). This difference may reflect physiologic adaptation, but could also reflect adaptation by selection to the Port Phillip Bay environment (which experiences greater salinity, temperature, and nutrient input extremes than Bass Strait) over the 8,000 y since the Bay was formed. The lack of significant differences between the two groups (Port Phillip Bay and Bass Strait) of *H. laevisgata* populations in the present study suggests that none of the molecular markers used are subject to selection themselves, or on closely linked loci, and that the differences observed are likely to reflect sampling effects associated with high variance in reproductive success between matings and founder effect. However, this data does not preclude adaptation to the Port Phillip Bay environment.

Ranching Implications, Recommendations, and Future Work

The genetic diversity of the *H. laevisgata* population assimilated for a ranching trial was at the lower end of the range for other populations sampled. The differences observed between this population and others in Port Phillip Bay are consistent with a recent founder effect or variance in reproductive success. This suggests it should be feasible to add diversity to the ranching population using individuals collected from elsewhere. Given the possibility that Port Phillip Bay animals may differ physiologically from those at

ocean sites it would be prudent, until more information on this point is available, to use only animals from Bay sites. Fifty to 100 abalone broodstock of equal sex ratio need to be used to produce juveniles to maintain genetic diversity (Withler et al. 2001). It has been shown that mass matings do not necessarily ensure a large number of adults contribute to the next generation (Hedgecock & Sly 1990). It will also be important to manage breeding of the Point Cook population to avoid mating close relatives. Therefore, methods of single pair mating may need to be developed, and the population will need to be monitored to ensure genetic management objectives are met.

ACKNOWLEDGMENTS

The authors thank Dr. Nick Robinson and Matt Baranski from the Victorian Institute of Animal Science, for provision of primer sets *Hrub4* and *Hrub12*; Darren James and Wayne Butler for assistance with collecting fresh tissue samples; Tony Smith (Bay City Seafarm), Kaz Bartaska (Lonimar Australia Pty Ltd), and Fred Glassbrenner (Australian Abalone Exports Pty Ltd), for providing samples of live abalone tissues. The authors also thank two anonymous reviewers and Dr. Chris Burrige from Deakin University, whose comments improved the quality of this manuscript.

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MEASURING ABALONE (*HALIOTIS* SPP.) RECRUITMENT IN CALIFORNIA TO EXAMINE RECRUITMENT OVERFISHING AND RECOVERY CRITERIA

LAURA ROGERS-BENNETT,^{1,2*} BRIAN L. ALLEN^{1,3} AND GARY E. DAVIS⁴

¹California Department of Fish and Game, Bodega Bay, California 94923; ²U.C. Bodega Marine Laboratory, PO Box 247, Bodega Bay, California 94923; ³Squaxin Island Tribe, 2952 S.E. Old Olympic Hwy, Shelton, Washington 98584; ⁴Channel Islands National Park, 1901 Spinnaker Dr., Ventura, California 93001

ABSTRACT Abalone populations in southern California have declined dramatically since the 1950s when they supported a multi-species, commercial, and recreational fishery producing more than 3,000 t per year. Today the commercial fishery is closed statewide and the recreational fishery is closed south of San Francisco. In contrast, red abalone, *Haliotis rufescens* (Swainson, 1822), populations in northern California continue to sustain a 1,100 t per year free-diving recreational fishery. We used standardized Abalone Recruitment Modules (ARMs) made of half cinder blocks (area = 2.6 m²) to compare the recruitment of juvenile abalone in northern California, where stocks are abundant, with southern California where stocks have declined. We compared the abundance of abalone inside ARMs ($n = 12$) in Van Damme State Park (VDSP), northern California with abalone inside ARMs ($n = 82$) in the Channel Islands National Park from 2001 to 2003. Abalone densities on the reefs surrounding the ARMs at VDSP, averaged 8300/ha compared with abalone densities of 30/ha on reefs in three of the northern Channel Islands. Red, flat abalone, *H. walallensis* and pinto abalone, *H. kamtschaticana kamtschaticana* were found in the northern ARMs, whereas in the south red, pink, *H. corrugata*, threaded, *H. kamtschaticana assimilis*, and the endangered white abalone, *H. sorenseni*, were rare in the southern ARMs. Abalone were 30 times more abundant inside the ARMs in the north (5.30/ARM) compared with the south (0.18/ARM). Similar numbers of abalone were found in all 3 years in the ARMs in northern (69, 69, and 53 abalone) and southern California (14, 11, and 20 abalone). The majority of abalone in both the north and the south were less than 100 mm in shell length. Ironically, the rare flat abalone was more abundant in the north, than pink abalone were in the south, a species which once supported a major fishery. Clearly, abalone stocks in southern California are so low that recruitment is failing, despite their potential high fecundity and the fishery closure. These results demonstrate that ARMs can be used to monitor recruitment in the northern fishery, as well as establish quantitative recovery criteria to assess abalone restoration efforts that are desperately needed in the south.

KEY WORDS: juvenile abalone, abalone recruitment modules, monitoring, recruitment failure, abalone restoration

INTRODUCTION

Abalone populations in California once supported major fisheries, landing in excess of 3000 metric tons (t) per year (Cox 1962). Population declines, however forced the closure of both the recreational fishery south of San Francisco and the commercial fishery statewide in 1996. Populations in the south suffered serial depletion of five species within the *Haliotis* spp complex (Dugan & Davis 1993, Karpov et al. 2000). The white abalone, *H. sorenseni*, is now on the endangered species list and the primary cause of their decline has been attributed to overfishing (Hobday et al. 2001). In contrast, red abalone, *H. rufescens*, populations are still abundant in northern California where there is an active free-diving recreational fishery. Landings in the recreational fishery have been stable, averaging 1165 t in the mid 1980s (Tegner et al. 1992) with little change in 2000 (1238 t) (CDFG unpubl. data). Today, there is a need to quantify juvenile abalone recruitment in California to better manage fished populations in the north and to assess the status of depleted stocks in the south since the fishery closure. Specifically, temporal patterns of juvenile abundance can be used to assess the strength of future year classes entering the fishery as well as set restoration targets for populations following natural recovery or restoration efforts.

Recruitment of abalone, however, is difficult to quantify. Studies of larval dispersal and recruitment are rare (but see Sasaki & Shepherd 1995), with only a handful of studies examining the density and distribution of newly settled abalone (Prince et al. 1987, McShane & Smith 1991, Sasaki & Shepherd 2001). An

examination of densities of newly settled abalone (recruiting to the benthos) has been advocated for to quantify stock-recruitment relationships and density dependent survival, but these studies are impeded by the difficulties of sampling (McShane & Smith 1988). Most recruitment studies focus on the abundance of juvenile or young of the year abalone, sometime after settlement, when they are visible to divers (Sainsbury 1982, Shepherd & Turner 1985, Prince et al. 1988). Juveniles may be better predictors of the numbers of abalone surviving to become adults as compared with larval and newly settled abalone. Juveniles however, are also difficult to census (Yamaguchi 1975) sometimes requiring habitat destruction especially on complex rocky reefs.

Artificial structures made of cinder blocks have been used as standardized sampling units (Davis 1985) for juvenile abalone in southern California (Davis 1995). However, few juveniles have been found within these modules suggesting either recruitment failure, as is consistent with reef surveys showing little recruitment and few adults, or the modules are poorly suited to assess juvenile abalone in the wild. Recruitment overfishing may be difficult if not impossible to quantify (Harrison 1986, McShane 1992) because a sharp decrease in recruitment in species such as abalone with high individual fecundities may not occur (Harrison 1986).

Here we compare the numbers of juvenile abalone inside standardized recruitment modules made of cinder blocks deployed in northern California where densities of abalone are high, with southern California where densities are low. We examine the species composition, quantity, and size of juvenile abalone occurring inside abalone recruitment modules in 2001 and 2003. We quantify abalone populations on the natural rocky reefs surrounding the recruitment modules in both the north and south and compare them

*Corresponding author. E-mail: rogersbennett@ucdavis.edu

with the abalone inside the modules. We highlight the usefulness of collecting a time series of juvenile abalone recruitment information in California to aid in fishery management and evaluate the impacts of restoration programs. We examine the current status of abalone recruitment in southern California by comparing modern densities in the southern modules with the north and the south 10 years ago. We discuss the evidence for recruitment failure in southern California and the need for establishing measurable restoration goals.

MATERIALS AND METHODS

Northern California

Standardized abalone recruitment modules (ARMs) were deployed at Van Damme State Park (VDSP) (lat. $39^{\circ}16'08''\text{N}$, long. $123^{\circ}47'58''\text{W}$), northern California in August 2000 (Fig. 1). ARMs

were originally designed by E. Ebert and were made of concrete cinder blocks cut lengthwise, stacking the blocks five high and enclosing them in a wire frame (Davis 1995). Here, the design was modified to enhance resistance to intense wave action by using welded rebar cages, widening the block cube to lower the profile, stacking the blocks three high (rectangle measuring $813\text{ mm} \times 610\text{ mm} \times 305\text{ mm}$ high). The surface area of the blocks in each of the modified northern ARMs was 4.8 m^2 , of which 2.6 m^2 was sheltered area created by the sides, top, and bottom of each "letter m."

VDSP is a popular and productive recreational abalone diving site dominated by rocky reef with seasonally abundant *Nereocystis* kelp canopy, a subcanopy of *Pterogophora* and numerous subtidal red algae including *Cryptopleura*, *Gigartina*, and *Iridaea*. Upright and encrusting coralline algae are also abundant. An estimated $25,500 (\pm 5,430)$ red abalone were taken from VDSP in 2002 (CDFG unpubl. data).

Twelve ARMs were deployed between 9 and 12 m in three



Figure 1. A map of California showing sites in both northern and southern California where ARMs were deployed and abalone recruitment was monitored.

groups of four. The modules were placed 3–6 m apart within the cluster of four. The northern cluster was approximately 140 m from the central cluster. The southern cluster was 710 m from the central cluster. Each ARM was bolted into the rocky substrate using 6-inch stainless lag bolts in each of the four corners and in the center of the module. Holes for the 5 bolts were drilled using a Chicago pneumatic drill with a 0.25 inch bit.

The ARMs were surveyed annually in August. ARMs were in place 1 y before the surveys began in August 2001, 2002, and 2003. One dive team surveyed each ARM, opening the lid and carefully removing each block counting and measuring all of the invertebrates and fishes inside the ARMs. Organisms outside or on the cage were not sampled. Organisms too numerous to count were gently swept into fine mesh bags (2 mm) to be counted and measured at the surface. Organisms cemented to the brick were measured and quantified underwater. Each block was set to the side while the next block was removed and the animals quantified. When the survey was completed, divers reassembled the ARM and replaced the lid securing it with wide cable ties. Each ARM took two divers approximately 40 min to survey.

Abalone density on the adjacent natural reef was quantified annually in August from 2000 using transects at depths between 8 and 16 m. Each transect (1m × 5m) was searched invasively for abalone in 2000 ($n = 29$), 2001 ($n = 13$) and 2002 ($n = 32$). Invasive searching targeted small cryptic abalone and included looking in rock crevices, turning large rocks and small cobble, in coralline algae, and under sea urchins.

Southern California

ARMs have been deployed and surveyed by the National Parks Service's, Kelp Forest Monitoring Program in the northern Channel Islands since 1989 (Davis et al. 1997). These ARMs were cube shaped and slightly taller and narrower than the ARMs deployed in northern California (see details of the modifications required for the northern modules above). Each cage is made of plastic coated wire mesh (51 mm × 102 mm). The southern modules measured 600 mm × 600 mm × 500 mm high. Twenty half-cinder blocks were arranged four in a layer and stacked 5 layers high leaving the center core of the cube open. Each habitat provided 5.0 m² of which 3.0 m² was sheltered habitat created by the inside arches of the letter "m" which is comparable to the area of the northern California modules. The major differences between the southern and the northern module design was that the northern design had a lower profile, 18 half blocks and was bolted into the substrate whereas the southern modules were taller and more cube shaped, with 20 half blocks, an open core and were not bolted in place.

In 2001, 82 ARMs were sampled from three sites on Anacapa Island, five sites on Santa Cruz Island and two sites on Santa Rosa Island using the methods described earlier (Fig. 1). Each site comprised between 6 and 15 ARMs. Sites with 15 ARMs had three clusters of 5 ARMs each; at sites with only 6 or 7 all ARMs were grouped together in a single cluster. The majority of these sites were on the southern leeward side of the islands. In 2002, 84 ARMs were sampled and in 2003 at the same sites and islands 82 ARMs were sampled.

The density of adult red, pink, and green abalones were enumerated along transects on the natural reef. Adult abalone abundances were determined at each of the three islands with the ARMs. Depths of the transects at the three islands ranged from 5–16 m at Anacapa, 8–18 m at Santa Rosa and 4–18 m at Santa

Cruz Island. Deep-water species such as white and threaded abalone would not be found at these depths nor would shallow green abalone. A fixed reference line 100 m in length was bolted to substrate. Transects were placed perpendicular to this fixed reference line at random intervals. Transects covered an area 3 m × 20 m (60 m²). Twelve transects were completed at each site. Three sites were monitored at each of Anacapa Island (total area searched 2,160 m²) and Santa Rosa Island (2,160 m²) whereas 5 sites were monitored at Santa Cruz Island (total 3,600 m²).

The number of ARMs or replicates needed to detect "true" differences in the mean number of abalone in the north compared with the south was estimated using the test for sample size and the iterative method (Sokal & Rohlf 1981).

RESULTS

Abalone in the ARMs

Many more abalone were found inside the ARMs in northern compared with southern California in all 3 y (2001–2003) (Tables 1, 2). The number of abalone found inside the ARMs was consistent over the 3-y period (Tables 1 and 2). In the north 5.3 abalone were found per ARM. This was 30 times more abalone than observed in the south where the average was 0.18/ARM. In the north, three species of abalone were found inside the modules red flat and pinto abalone, whereas in the south, three species and one subspecies of abalone were found including two deep-water abalone (white and threaded abalone), albeit in very low numbers. In southern California, red, pink, white, and threaded abalone were found inside the ARMs in 2001–2003. In previous years, green abalone, *H. fulgens*, have also been observed in the ARMs (5 total in 10 y).

There was an 80% chance of detecting at least a 10% difference in the means of juvenile abalone density in northern compared with southern California at the 0.05 significance level with a sample size of 9 or more ARMs in each region.

Abalone on the Natural Reef

Abalone density on the natural reef surrounding the ARMs differed substantially between northern and southern California (Table 3). In northern California, abalone were found at high densities, while in southern California densities were near zero. Abalone density in the north averaged nearly 8500 abalone/ha (SD 2,302/ha) between 2000 and 2002 (Table 3). Of the 679 abalone measured in the north, 669 were red abalone, 9 were flat abalone and 1 was a pinto abalone (Table 3). Furthermore, abalone were abundant both shallower and deeper than the surveys. The density

TABLE 1.

Number and species of juvenile and adult abalone found within ARMs in Van Damme State Park, northern California in Aug. 2001, 2002, and 2003. One pinto abalone 83 mm was found in 2003.

Year	# ARMs	<i>H. rufescens</i>		<i>H. walallensis</i>		Total
		<50 mm	>50 mm	<50 mm	>50 mm	
2001	12	36	17	4	12	69
2002	12	25	25	3	16	69
2003	12	17	15	3	17	52
Total	36	78	57	10	45	190

TABLE 2.
Number and species of abalone inside ARMs southern California in 2001 to 2003.

Island	#ARMS	<i>H. rufescens</i>	<i>H. corrugata</i>	<i>H. sorreusci</i>	<i>H. k. assimilis</i>
2001					
Anacapa Is.	20	0	2	0	0
Santa Cruz Is.	47	4	1	1	4
Santa Rosa Is.	15	2	0	0	0
Subtotal	82	6	3	1	4
2002					
Anacapa Is.	20	0	1	0	0
Santa Cruz Is.	48	1	0	0	5
Santa Rosa Is.	16	4	0	0	0
Subtotal	84	5	1	0	5
2003					
Anacapa Is.	20	0	1	0	0
Santa Cruz Is.	46	1	0	0	8
Santa Rosa Is.	16	10	0	0	0
Subtotal	82	11	1	0	8
	248	22	5	1	17

of flat abalone was higher in the ARMs (4.8 abalone m^{-2}) than on the surrounding natural reef (0.012 abalone m^{-2}) in the north.

Few abalone were observed during the surveys at the three Channel Islands. No abalone were observed at Santa Cruz Island on any of the surveys. In the south, white and threaded abalone were found inside the ARMs but were absent from the shallow surveys of surrounding reef, at the depths surveyed (Tables 2 and 3). At Anacapa Island only 7 pink abalone were observed over the 3-y period during which 6,480 m^2 were searched. At Santa Rosa

Island abalone densities were slightly higher with 20 red abalone observed (31/ha) in the same area searched (Table 3). In the south, the numbers of adult abalones were consistently low, with abalone found at a small fraction (3/11) of the total number of sites.

Size Frequency Distributions

Small (<50 mm) and large abalone were found inside the ARMs in both northern and southern California. In the north, a

TABLE 3.
Average density of abalone, *Haliotis* spp. found in the vicinity of ARMs in northern and southern California from 2000 to 2002. Density in northern California was determined along 2 m \times 5 m (10 m^2). Density in southern California was determined along 3 m \times 2 m (60 m^2). Abalone in northern California included red, flat and pinto abalone with red and pink abalone in southern California.

Site	Year	Area m^2	Count	Density/ m^2	Density/Ha.
North					
Van Damme State Park	2000	290	320	1.10	11,034
	2001	130	71	0.55	5,462
	2002	320	288	0.90	9,000
Average		247	226	0.85	8,499
South					
Anacapa Island*	2000	2,160	2	0.001	9.26
	2001	2,160	3	0.001	13.88
	2002	2,160	2	0.001	9.26
Average			2.67	0.001	10.80
Santa Cruz Island	2000	3,600	0		
	2001	3,600	0		
	2002	3,600	0		
Average			0		
Santa Rosa Island**	2000	2,160	15	0.007	69.44
	2001	2,160	2	0.001	9.26
	2002	2,160	3	0.001	13.88
Average			6.67		30.86

Note: *All abalone found at Anacapa Island were pink abalone, *H. corrugata*.

**All abalone found at Santa Rosa Island were red abalone, *H. rufescens*.

peak in young of the year abalone (<30 mm) was observed in 2001, but this peak was less pronounced in 2002 when more midsize juvenile abalone (50 mm) were observed in the size frequency distribution (Fig. 2). In the south, all the abalone observed in the ARMs were small except one adult (Fig. 2).

Qualitatively, the shape of the size frequency distribution at the small end (left side) was relatively similar in 2002 inside the ARMs compared with on the natural reef. This was not the case in 2001 when more juveniles in the 20-mm size class appeared in the modules than in surveys of the natural reef (Fig. 3). Overall, many more adult abalone (>100 mm) were observed on the transects over the natural reef compared with the ARMs suggesting the ARMs are not suitable for sampling adults. In southern California, too few adult red ($n = 20$) and pink abalone ($n = 7$) were observed on the natural reef to warrant comparing their size distribution with the sizes of the juveniles inside the ARMs.

DISCUSSION

The dynamics of abalone recruitment and the nature of the stock-recruitment relationship remains poorly understood, yet has important implications for management and restoration. In this study, we found recruitment failure (0.18/ARM) in southern California where adult densities are two orders of magnitude below the proposed minimum viable population size (2,000/ha) (Shepherd & Brown 1993). Furthermore, we found that abalone recruitment in southern California has declined 20-fold over the past decade, despite the fishery closure. In 1990 ($n = 161$) and 1991 ($n = 154$) densities inside 45 ARMs at Santa Rosa and Santa Cruz Islands in the Channel Islands National Park (Davis 1995) were higher than

today (3.6/ARM). If conditions remain unchanged in the south, we may no longer be able to detect recruitment using <100 ARMs. This suggests that adult densities may have declined to the point where we are now observing recruitment overfishing.

In contrast, to the south higher numbers of juvenile abalone (5.30/ARM) were found in northern California from 2001 to 2003 where adult red abalone densities were 8,000/ha on the natural reefs. Similar numbers of juveniles in the north were found in the ARMs as compared with the natural reef (Fig. 3), however too few abalone were observed to make this comparison in the south. Elsewhere higher densities have been found inside artificial habitats than on the natural reef (Hayashi & Yamakawa 1988, DeFreitas 2003). The endangered white abalone (Hobday et al. 2001), the pinto, and threaded abalone (Rogers-Bennett et al. 2002a) were all found in ARMs indicating they are suitable for monitoring even rare species. In the north, flat abalone were regularly observed inside ARMs even though they make up less than 14% of the population (Rogers-Bennett & Pearse 2001). Our results suggest ARMs are a useful tool for monitoring juvenile abundance in an active fishery as well as where populations have declined. The numbers of juveniles within ARMs from healthy stocks in the north (5.3/ARM) can be used as minimum targets for restoration criteria in California.

We examined the abundance of juvenile abalone, some months after settlement as previous studies have done (Sainsbury 1982, Shepherd & Turner 1985, Prince et al. 1987). To assess year class strength, a time series of these data could be collected encompassing a variety of oceanographic conditions including high-frequency environmental fluctuations such as El Niños, as well as low frequency environmental fluctuations such as regime shifts

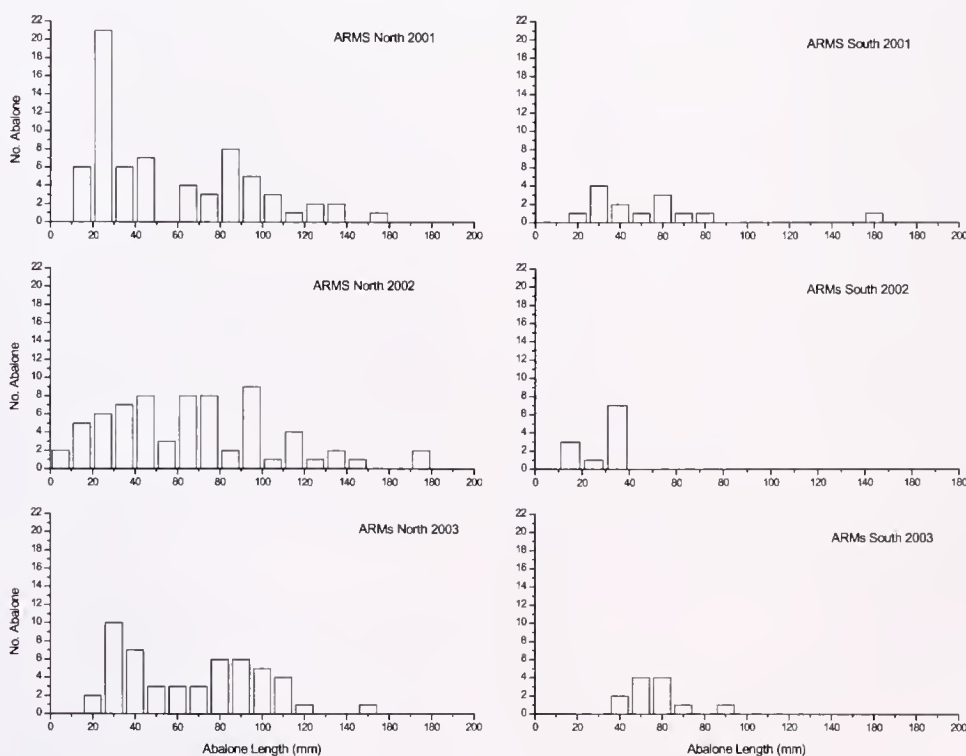


Figure 2. A comparison of the size frequency distributions of abalone *Haliotis* spp. found inside ARMs in southern and northern California in 2001, 2002, and 2003. The top two graphs are the abalone found in the ARMs in southern California and the bottom two graphs are the abalone found in the ARMs in northern California. The length of the abalone in mm is on the X axis and the number of abalone of that size is shown on the Y axis.

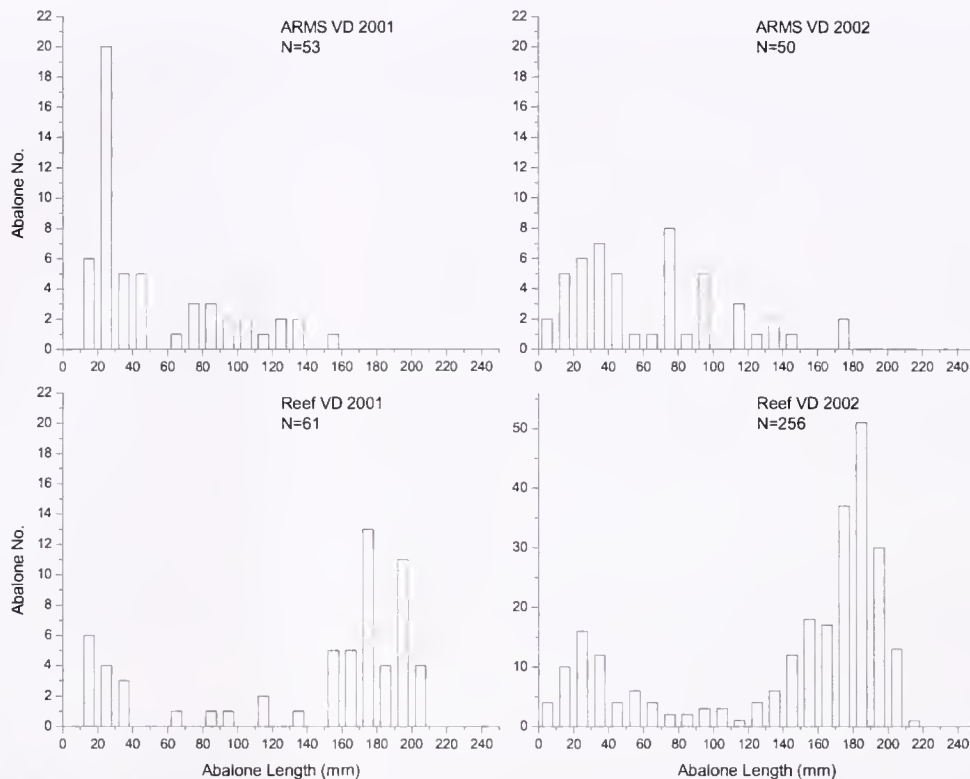


Figure 3. A comparison of the size frequency distribution of red abalone, *H. rufescens*, found inside ARMs and on the natural reef in Van Damme State Park, northern California in 2001 and 2002. No abalone measurements were made on the natural reef in 2003. The top two graphs are abalone found inside the ARMs and the bottom two graphs are abalone found on the natural reef.

(Mantua et al. 1997). Furthermore, recruitment levels could be assessed across a gradient of adult densities, in between the high and low densities that we have examined, to reveal the relationship between stock size and recruitment.

Establishing if there is a relationship between spawners and recruits at the local level is vital for protecting against localized recruitment overfishing. In southern Australia, where the longest time series of stock-recruitment measures exist for abalone (examining 2-y-old juveniles), no clear relationship was found in a reserve population (Shepherd 1990). Experimental adult removals however, demonstrated that there was a positive correlation between local adult density and the number of newly settled abalone (Prince et al. 1988). Either adult spawner density is linked with local recruitment, or larvae from local and distant sources preferentially settle and survive with high densities of adult abalone (Breen 1992). Abalone unlike other invertebrates, such as sea urchins, may have limited larval dispersal (Tegner & Butler 1985, Prince et al. 1987). This suggests restoration programs should not rely on natural larval dispersal to recover distant populations (Tegner 1993). A minimum density threshold (allee effect), has been suggested for abalone (Shepherd & Brown 1993) below which recruitment fails precipitating either local or global extinction (Roberts & Hawkins 1999). Our results in the south support this threshold concept. One mechanism proposed to explain this threshold is the significant drop in fertilization success at low densities despite high fecundities (Babcock & Keesing 1999). This is relevant not only for management of the recreational red abalone fishery in the north, but also for restoration of endangered and threatened species of abalone in southern California.

In conclusion, recent abalone population surveys (2002) in

southern California indicate that 98% of the remnant pink, green and white abalone populations are comprised of large old adults (CDFG unpubl. data). The absence of large juveniles and small adults from the size frequency distributions suggests that there has been little successful recruitment on these natural reefs in the past decade (except for red abalone at San Miguel Island) (Rogers-Bennett pers. obser.). Densities of pink abalone are low even inside reserves in southern California (Rogers-Bennett et al. 2002b). Today, the white abalone in southern California is at dangerously low population levels and the absence of small adults and juveniles suggest there has been 2 decades of recruitment failure (Hobday et al. 2001, Lafferty et al. 2004). Given the dire state of many abalone populations in southern California today abalone restoration programs cannot delay. We recommend that abalone restoration programs (1) incorporate strategies to assess juvenile recruitment (such as ARMS); (2) determine recruitment levels based on healthy stocks; and (3) use measurable quantitative recovery criteria (Gerber & Hatch 2002).

ACKNOWLEDGMENTS

The authors thank D. Kushner for his assistance with the KFMP ARMs and transect data. The authors also thank all the CDFG and U.C. divers who helped install and census the ARMs along the ever challenging north coast. Special thanks to the CDFG dive program, the office in Fort Bragg, and wildlife protection. Support came from the recreational abalone stamp through the Recreational Abalone Advisory Committee and the California Department of Fish and Game. This manuscript was improved by two anonymous reviewers. Contribution number 2229, Bodega Marine Laboratory, University of California Davis.

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EFFECTS OF EXTENSIVE SEEDING ON ABALONE, *HALIOTIS DISCUS DISCUS*, ABUNDANCE ON THE PACIFIC COAST OF BOSO PENINSULA, JAPAN

TOSIATU SIMIZU* AND KANAKO UCHINO

Chiba Prefectural Government, Fisheries Research Center, Japan

ABSTRACT Abalone resources in Chiba Prefecture, the Pacific coast of central Japan, have been severely reduced in recent years. Our investigations have revealed that the density of the mature abalone in this area is 0.019 ind./m², which may be insufficient for successful reproduction. In this study, we investigate the effects of extensive seeding on stock enhancement of abalone, *Haliotis discus discus*. We experimentally released 400,000 juveniles (3.0 cm in shell length, SL) to the research field of 8,000 m² in May 1998 and investigated the growth and stock conditions by SCUBA. The abalone seeds grew up to 10.2 cm SL on average in 3 y and 12.8 cm SL in 4 y, exceeding the legal catch size (12.0 cm) by the Chiba Prefectural Regulation of Fisheries Adjustment, and subsequently commercial catches started in 2002. The catch per unit effort (CPUE) was increased to a great extent from 3 kg/day-boat to 9 kg/day-boat. The inhabited area was considered to be expanded to 100,000 m² based on the catch logs by the local fishermen. As of May, 2002, a production of 57,000 abalones was estimated to be attained by the extensive seeding.

KEY WORDS: seeding, stock enhancement, abalone

INTRODUCTION

Abalone catch used to be 800 t a year in Chiba Prefecture, the Pacific coast of central Japan. The catch has been remarkably decreased to less than 100 t in recent years. Our investigation has revealed that the density of mature abalone is 0.019 ind./m², which may be insufficient for successful reproduction (Shepherd & Brown 1993, Shepherd & Partington 1995, Badcock & Keesing 1999). In this study, we investigate the effects of extensive seeding on stock enhancement of abalone, *Haliotis discus discus*.

METHODS

We experimentally released 400,000 artificial abalone juveniles (3 cm in shell length, SL; 4.3 g) to the research field of 8,000 m² in May 1998 and investigated the growth and density by SCUBA. The juveniles were produced from natural adults collected in the research field and were 1.5 y old at the time of release. The research field is "reefy" seashore (3–5 m in depth) and is one of abalone fishery grounds in Boso Peninsula.

The released abalone reached the legal catch size, and commercial catches started in May 2002. We analyzed the catch number and location data on the daily catch log recorded by all local fishermen (9 in total). The fishermen dived to catch the abalone with goggles and a bathing suit. It was prohibited for Ama fishermen to use a snorkel tube, fins, wetsuit, and SCUBA gears. The fishing season was from May to August.

The stock size was estimated by the DeLury method, which is based on the regression between CPUE and cumulative catch number. The occurrence rate of released abalone out of the total catch was multiplied to the estimated stock size to obtain the stock size of the released abalone. Released abalone can be easily recognized by greenish color of the shell; however, because the recognition results differed between fishermen and us, we used our results.

The horizontal distribution of the released abalone was estimated with the estimated stock size and the CPUE values calculated for each 50-m transects.

The survival rate of released abalone seeds is supposed to be low within the first 1 mo after releasing and become higher and stable afterwards (Tanaka & Sakamoto 2001). We set the stable natural mortality coefficient to be 0.223/year (Simizu 2000), and the early survival rate was set to be 0.33, which was calculated with estimated stock size and the number of released shells.

The expected catch of released shells in weight and price within 15 y after releasing were evaluated by growth-survival model (Beverton & Holt 1956), and the price values were compared with the expenditure, which is required for seeding. The average fishing mortality values (0.184) was used for the evaluation.

RESULTS AND DISCUSSION

Growth and Maturation

The abalone seeds grew up to 10.2 cm SL on average in 3 y and 12.8 cm SL in 4 y, exceeding the legal catch size (12.0 cm) by the Chiba Prefectural Regulation of Fisheries Adjustment (Fig. 1). Because the size at the time of release (1.5-y-old) was small compared with natural abalone (Simizu 2000, 2001), subsequent growth was also slower. Testes of 4 males (mean SL = 7.3 cm) were in mature stage (Tomita 1968), and ovaries of 4 females (mean SL = 7.5 cm) were in premature stage or mature stage (Tomita 1967) in October 1999 (Fig. 2). We observed that some abalones recaptured in May 2002 and kept in a tank spawned eggs in following November.

Habitation

Our SCUBA investigations revealed that the abalone densities, which were 50 ind./m² at the time of release, were 0.17 to 0.20 ind./m² 1.5–3 y after the release, and 0.45 ind./m² 4 y after the release. According to the analyses based on the catch logs by the local fishermen, the densities were 0.56 ind./m² on average, and the maximum was 1.8 ind./m² 4 y after the release. Higher density values in 4 y rather than in 1.5 y after the release was probably because of the difficulty in finding smaller individuals hiding between rocks. The habitation range expanded to 10,000 m² in May 2002 (i.e., 4 y after the release) shoreward toward shallow areas

*Corresponding author. E-mail: t.shmiz7@mb.pref.chiba.jp

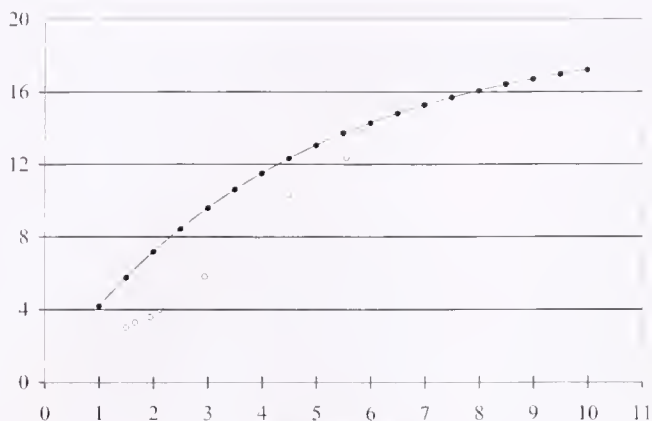


Figure 1. Growth comparison between seeding abalone and natural abalone *Haliotis discus discus*. The horizontal axis shows the age, and the vertical axis shows shell length in cm. Open circle shows seeding abalone. Solid circle shows natural abalone.

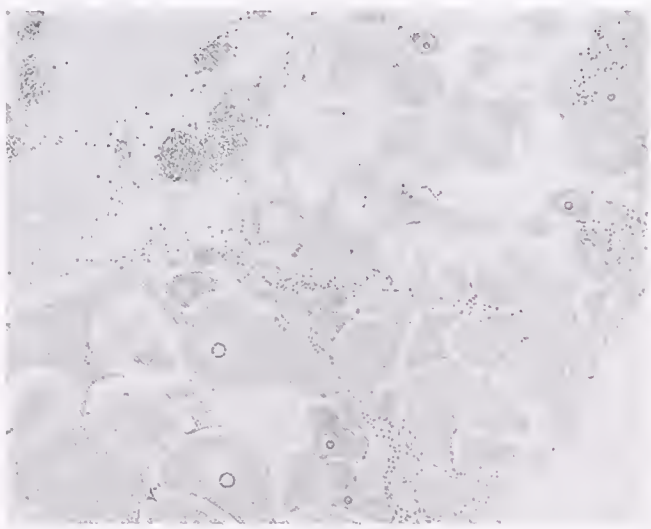


Figure 2. Section image of immature female gonad of *Haliotis discus discus*, collected in October 1999. (7.5-cm shell length).

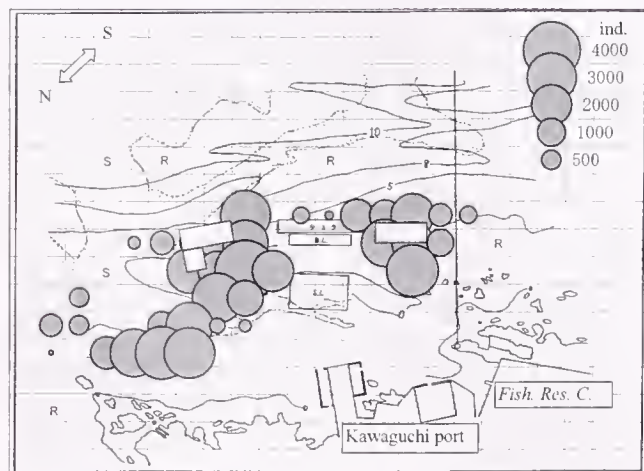


Figure 3. Distribution pattern of released abalone, *Haliotis discus discus*, in May 2002. Estimation is based on local fishermen's daily log data. Open squares indicate the release sites.

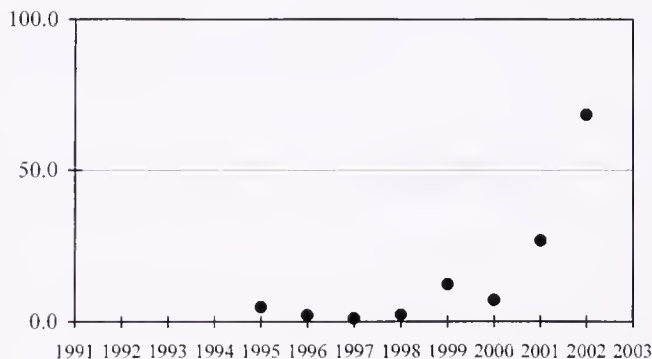


Figure 4. Occurrence of released abalone in landings. Vertical axis shows % occurrence rate of released abalone out of the total landings.

with many stones (Fig. 3). This may be because seabed with many stones is more suitable than reefy bottom as a habitat.

The Catch and Production of Abalone Resources by Seeding

Because the seedlings had not been done in the survey fishery ground, occurrence of released abalone was low. However, it increased rapidly to 68% in 2002 (Fig. 4). Increased number of released abalone increased CPUE to a great extent from 3 kg/day-boat to 9 kg/day-boat (Fig. 5).

The number of abalone was greatly reduced from 400,000 to 132,000 in 1 mo after the release in May, 1998, and it became 57,000 in May 2002 (Fig. 6). A part of the abalone with fast growth was caught in 2001 (1,600 individuals), and all individuals recruited to fishing in May 2002. During the fishing season in 2002, 4,500 individuals were caught.

Trial Calculation of Seeding Effects

The overall recapture landing was provisionally calculated to be 31,000 individuals or 10.4 t. Recapture rate was calculated to be 7.8%. Seeding used to be conducted in the survey area from 1968 to 1981, and the recapture rate during this period was estimated to be 8.8% (Sakamoto *et al.* 1986). The average unit price of the abalone is assumed to be 6,200 yen/kg, thus the effect of seeding is calculated to be 65 million yen. The expenditure of seedlings was 19 million yen; therefore, substantial benefit is considerable

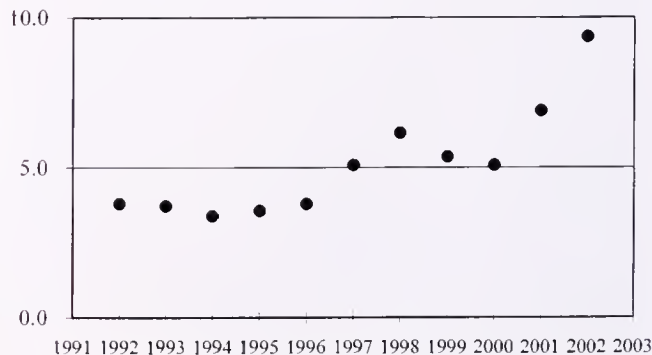


Figure 5. Estimated distortion of CPUE (kg/day-boat) from 1992 to 2002. The horizontal axis shows the year, and the vertical axis shows CPUE.

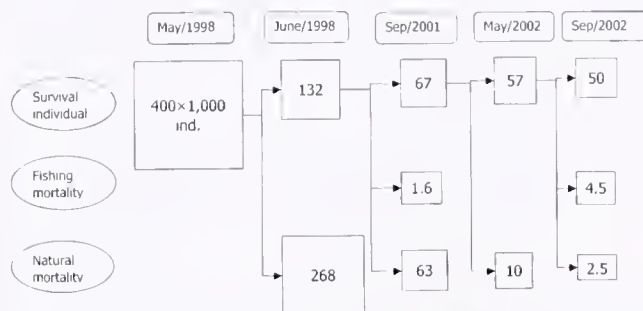


Figure 6. Survival process of released abalone seeds from May 1998 to September 2002.

even after handling and transportation expenses are subtracted. Continuous field survey is necessary to estimate accurate figures.

As of May, 2002, a production of 57,000 abalones was estimated to be attained by the extensive seeding. The maximum abalone density was estimated to be as much as 1.8 ind./m². These presumption results indicate effective enhancement of abalone production.

ACKNOWLEDGMENTS

The authors thank Dr. Toyomitsu Horii and Dr. Satoshi Watanabe, National Research Institute of Fisheries Science, for their critical reading of this manuscript.

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GONAD DEVELOPMENT IN SEEDED *HALIOTIS LAEVIGATA*: GROWTH ENVIRONMENT DETERMINES INITIAL REPRODUCTIVE INVESTMENT

LUKE A. MCAVANEY,* ROBERT W. DAY, CAMERON D. DIXON AND SYLVAIN M. HUCHETTE

Zoology Department, University of Melbourne, Victoria, 3010, Australia

ABSTRACT Cultured *Haliotis laevigata* (Donovan), 18 mo old, were tagged and seeded at either high or low densities on boulder reefs (4–6 m depth) at Cape Jervis, South Australia in September 2001. Surveys every 3 mo quantified growth history and a sample was sacrificed in September 2002, prior to the expected spawning season of *H. laevigata* from this area, to measure gonad development. This provided a unique opportunity to investigate the onset of reproductive investment in known age *H. laevigata* in the field. Forty-five percent of sampled abalone had developed gonad tissue by September 2002. Larger individuals were more likely to have initiated gametogenesis and tended to have larger gonads. Gonad development was most closely related to abalone weight in September 2002 and was also strongly correlated with length and early growth rate. A significant proportion of abalone migrated from cryptic positions within boulder habitats into more exposed adult habitat at the outer edge of reefs between April and June. Abalone that had emerged to adult habitat were significantly more likely to be mature than cryptic individuals. The overall proportion of abalone with developing gonads differed significantly between densities. This seems to be an indirect effect, arising because abalone at low density grew faster than those at high density. The onset of sexual maturation in *H. laevigata* was thus partly dependent on density and site characteristics, which probably reflect the relative availability and quality of food. The results suggest that onset of sexual maturity in *H. laevigata* can advance under conditions of fast growth. As sampled gonads contained ripe gametes and maturity appears to be related to growth rate, fast growing abalone may reproduce ≥ 1 y before smaller juveniles in the same cohort.

KEY WORDS: gonad development, growth, maturity, density, *Haliotis laevigata*

INTRODUCTION

Abalone exhibit wide variation in growth rate (reviewed in Day & Fleming 1992) and size at maturity (McShane & Naylor 1995a). Much of this variation is likely due to inherent variability in the environment, but other aspects may explain these patterns. Life history theory predicts that most organisms will mature at neither a fixed age nor at a fixed size, but along an age–size trajectory according to environmental constraints such that reproductive fitness is optimized (Stearns & Koella 1986). For abalone, factors such as density, previous growth history, and food availability (or quality) may promote large differences in growth rate and the timing of maturation. How these factors specifically relate to gonad development, however, remains poorly understood. The difficulty of monitoring cryptic juveniles in the field (McShane & Smith 1988) has largely limited research to laboratory studies.

Intraspecific competition in abalone, which are dependent on a limited supply of drift algae as food (Shepherd 1973), might be expected to result in reduced growth and/or fecundity at higher densities. Laboratory and aquaculture studies have shown that high stocking densities can have strong negative effects on the growth of juvenile abalone (Mgaya & Mercer 1995, Capinpin et al. 1999, Huchette et al. 2003). A field experiment involving adult *H. iris* in New Zealand showed that reproductive investment (measured as dry gonad weight) was independent of density (McShane & Naylor 1995b). Douros (1985) found that size-specific reproductive investment in *H. cracherodii* was increased at a site where density was reduced, relative to a control site. Density effects on reproduction have been shown in several terrestrial molluscs (Carter & Ashdown 1984, Baur & Baur 1992), but there seem to be no studies of these effects on the initial reproductive investment of animals as they approach maturity.

Strong size-dependent mortality rates in juvenile abalone (Shepherd & Breen 1992, Shepherd 1998) may shape selective

pressure such that the onset of maturity is linked with the attainment of a threshold size (McShane & Naylor 1995a, Worthington & Andrew 1997). Nash (1990, 1992), however, concluded that sexual maturity is determined by reaching a certain age, rather than size, in *H. rubra*. This is also argued for other abalone species (Shepherd & Laws 1974, Shepherd et al. 1991) and has strong theoretical support (Stearns & Koella 1986). There is some evidence that the age of first maturity in abalone may be a plastic trait that is influenced by growth rate (McShane & Naylor 1995a) but this is yet to be demonstrated in a known-age population.

This field study investigated the effect of growth history (i.e., previous growth) and local density on the timing and degree of maturation in seeded abalone reaching first maturity. Practical difficulties associated with relying on natural recruitment in the field were avoided by using tagged, cultured juveniles that were all the same age.

MATERIALS AND METHODS

Experimental Design

This study was carried out near Cape Jervis, South Australia (138° 06'E, 35° 36'S) between September 2001 and September 2002. Eighteen-month-old cultured abalone from Kangaroo Island Abalone hatchery with mean shell length 28 mm (SE = 3 mm) were tagged with polyethylene tags (Hallprint, South Australia). Abalone were allowed to recover from the tagging procedure for 5 days before being transported to the study area in ice boxes filled with damp hessian sacks.

Four sites at depths of 4–6 m were selected according to their proximity to seagrass habitat (*Amphibolis* and *Posidonia* spp.) and the presence of adult *H. laevigata* in low densities. Sites were semiexposed areas of flat sand substratum surrounded by seagrass with high epiphyte loads and were situated 20–30 m from natural abalone reefs. At each site, six 6 m² rectangular experimental reefs were assembled, arranged in a 12 m × 9 m grid, each 3 m apart. Each experimental reef consisted of two layers of medium-sized

*Corresponding author. E-mail: l.mcavaney@pgrad.unimelb.edu.au

boulders (20–40 cm across) collected from the surrounding area with intact natural faunal assemblages associated with them. Six stocking densities (9, 14, 18, 24, 30, and 45 abalone per m²) were randomly allocated among the experimental reefs at each site. The two highest densities at each site were designated as high-density treatments and the remaining four densities were designated as low-density treatments. Tagged abalone were seeded onto experimental reefs in open bags of plastic mesh from which they could easily disperse. The methods used during the seeding process are dealt with more fully in Dixon et al. (2005).

At 3, 6, 9, and 12 mo postseeding, surveys were made to quantify growth and mortality. The reefs were progressively dismantled and reassembled by divers, and an exhaustive search undertaken to find all abalone from each reef. The tag number and length (to the nearest 1 mm) were recorded *in situ*, to calculate growth over each 3-mo period (growth increment). The position of each recovered abalone was also recorded to determine if the individual was more likely to have been exposed to higher flow rates on the tops of rocks or more food at the outer edges of reefs. During surveys, we chose not to remove predators from experimental sites as we sought to create near-natural conditions.

In early September 2002 (Austral spring), a number of abalone that had moved off reefs at one site were collected to determine an appropriate sampling regimen. These animals were removed from the shell and visually assessed for the presence or absence of gonad tissue. In this pilot study, the minimum size of abalone with differentiated gonad was ~60 mm. Following the results of the pilot study, we visited each site for sampling in late September 2002, just before spawning is expected in *H. laevigata* from this area (Shepherd 1992). We targeted our collection to recover 30 individuals from high and low-density reefs. After an exhaustive search of each reef, we sampled animals that were larger than 60 mm and represented a range of sizes present at each site. An additional 14 abalone >60 mm were randomly collected from high and low-density reefs at one site for histologic examination of gonad tissue.

Following sampling, abalone were weighed (drained live-weight) to the nearest 0.1 g and shell length was measured to the nearest 0.2 mm. Following measurement, the soft tissues were shucked and photographed in dorsal view using a SLR camera with a macro lens. The photographs were used to score a visual gonad index (VGI) based on the progression of the early stages of gonad development following the criteria in Table 1. In developing a scoring system for the VGI, it was important to include a wide range of possible scores to allow good estimates of the error variance in the data. The VGI differs from other visual indices in this respect, since these systems are based on much coarser (4 or 5 point) scales (e.g. Kikuchi & Uki 1975, Ault 1986). A fine scale VGI was also better able to distinguish between samples at the early stages of gonad development.

Histologic samples of gonad tissue were stained with Mallory Triple Stain and examined under a high power microscope for the presence of well-developed gametes (after Giorgi & DeMartini 1977).

Statistical Analyses

The visual gonad index used to quantify gonad development in maturing abalone cannot be considered an interval scale, and so could not be used as a dependent variable in statistical parametric analyses. To study the relationships between length, growth rate,

TABLE 1.
Criteria for assessment of the visual gonad index (VGI) in *H. laevigata* from Cape Jervis, South Australia.

VGI	Criteria
0	No gonad visible, immature.
1	Gonad membrane apparent, beginnings of gonad on under-side of conical appendage.
2	Some gonad membrane on proximal side of stomach and visceral spire.
3	Gonad tissue apparent on conical appendage.
4	Gonad tissue apparent on visceral spire.
5	Gonad tissue thickening around visceral spire and proximal side of stomach.
6	Gonad tissue thickening on conical appendage.
7	Thick gonad tissue on proximal side of stomach.
8	Approximately 50% of visceral spire covered in gonad tissue.
9	Visceral spire covered in gonad tissue.
10	Approximately 50% of conical appendage covered in gonad tissue.
11	Thick gonad tissue covering entire visceral spire, stomach and conical appendage.

and gonad development, simple regressions were performed against VGI. Although there is no reason to expect a linear relationship against the index scale in these analyses, the regressions measure the strength of a trend.

Because a large proportion of sampled abalone had a VGI of zero, parametric analyses of these data were not appropriate. To study the effects of density and site on gonad development, abalone were grouped by size. The proportion of abalone in each group with a VGI > 0 and the mean length of the group were measured as new variables. The proportion of mature abalone per group with some gonad could then be analyzed as the dependent variable in an analysis of covariance with mean length of the group as the covariate and treatment and site as factors. Similarly, an analysis of variance was performed with the mean size of the group as the response variable. One site was excluded from these analyses because only one individual at that site had developed gonad.

$P < 0.05$ was regarded as significant in all statistical tests (0.001, highly significant).

RESULTS

The mean density of abalone on experimental reefs a year after release (September 2002 *in situ* survey) was 16 abalone per m² at high density (range 15–17) and 7 abalone per m² at low density (range 5–11). A *t*-test showed that these densities had remained significantly different after 1 y ($t_7 = 4.708$, $P = 0.002$). Table 2 shows the size distribution of our overall sample from high and low-density reefs. Overall, 45% of the individuals sampled after 1 y had developed visible gonad tissue (48% at low-density, 40% at high-density). The mean length and weight of these was 74.1 mm (SE = 4.3 mm) and 47.5 g (SE = 8.8 g) respectively.

Examination of the 14 abalone collected for histology showed that those with discernable gonad development had gametes present in all cases and that females with a VGI > 4 had eggs in advanced stages of vitellogenesis (large, rounded oocytes, >100 µm). All male gonads appeared to contain viable sperm, spermatozoa being densely packed in the lumen of tubules.

TABLE 2.

Size distribution of *H. laevigata* sampled for gonad analysis in September, 2002.

Density	Size Class (mm)					Mean Size (mm)
	60–65	65–70	70–75	75–80	80+	
HIGH	9	41	40	12	1	70.2
LOW	1	36	53	40	11	72.4

A regression of length at capture after 1 y against VGI gave a significant positive relationship ($r^2 = 0.196$, $n = 244$, slope = 0.443, $P < 0.001$; Fig. 1). Thus abalone with a higher VGI tended to be larger and, conversely, those that had not developed gonad or had small gonads tended to be smaller. Regressions were performed to relate growth history and size of these abalone to gonad development (Table 3). Density was not considered in these models.

Over all size classes, more abalone were mature at low density than at high density (48% and 40% respectively, Fig. 2). Animals at high density, however, formed a larger percentage of mature animals in the medium size classes (e.g. up to 13% more by the 72-mm class). In the analysis of covariance (described in the Methods section), density treatment showed no significant effect on gonad development, but the covariate of length was highly significant (ANCOVA: $F_{1,23} = 38.195$, $P < 0.001$). Furthermore, density had a significant effect on the mean size of abalone across all sites (2-way ANOVA: $F_{1,24} = 6.774$, $P = 0.016$).

There was a marked increase at all sites in the percentage of abalone that were found on the edge of experimental reefs (i.e. adjacent to sand and seagrass) between March and June (Fig. 3). Abalone on the edge of the reef in June were also more likely to have developed gonad by September (2-way table: $\chi^2 = 5.881$; $P = 0.015$). Being on the edge of the reef at other survey times did

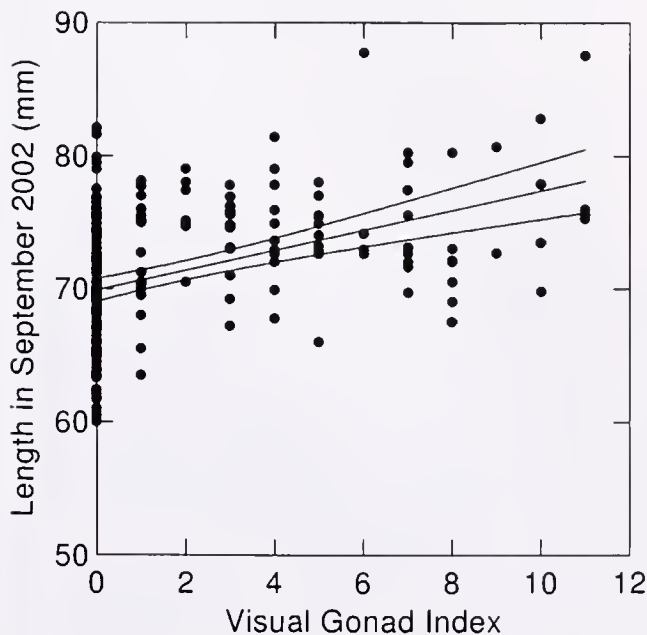


Figure 1. Plot of length at capture against visual gonad index for *H. laevigata* from Cape Jervis, South Australia. Data from all sites and reefs are pooled. Regression line is fitted with 95% confidence interval.

TABLE 3.

Regression for growth, length and weight measurements against visual gonad index for *H. laevigata* from Cape Jervis, South Australia.

Measurement	r^2	Slope
<i>Length</i>		
September 2001	0.006	—
December 2001	0.135	0.367
March 2002	0.139	0.373
June 2002	0.165	0.407
September 2002	0.196	0.443
<i>Length Increment (growth)</i>		
September to December 2001 (Spring)	0.132	0.363
December 2001 to March 2002 (Summer)	0	—
March to June 2002 (Autumn)	0	—
June to September 2002 (Winter)	0.032	0.180
<i>Weight</i>		
September 2002	0.224	0.473

not correlate with gonad development. Abalone found on the tops of rocks during the study were also no more likely to develop gonad than other animals. A smaller proportion of individuals were on the edge of reefs at high density compared with low-density reefs (Fig. 3) and this difference was significant in June (2-way table: $\chi^2 = 5.14$, $P = 0.023$).

DISCUSSION

This study was carried out using 18-mo-old cultured juveniles (28.1 mm mean length) that were seeded into a near-natural field environment. Growth rates for these animals while in the hatchery (i.e. prior to release) were very similar to those reported for wild

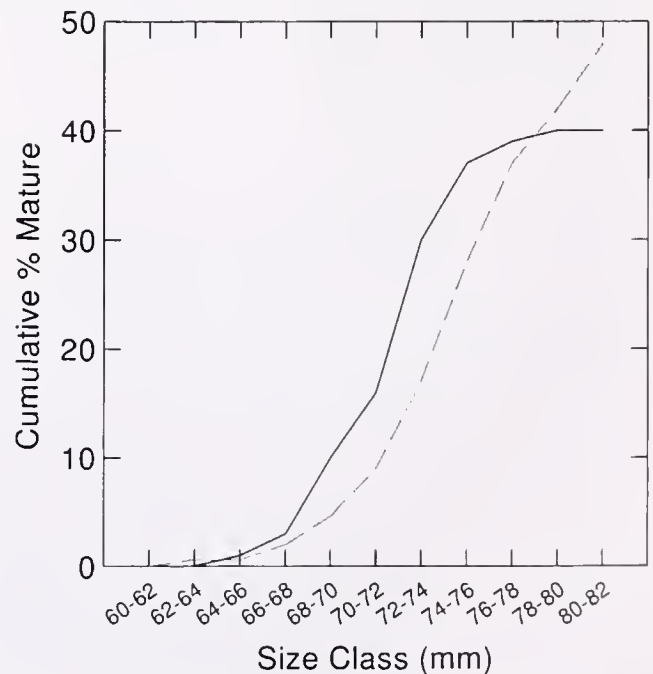


Figure 2. Line graph showing the cumulative percentage of *H. laevigata* with gonad tissue for each density treatment. Data from all sites are pooled. Solid line = High density; Dashed line = Low density.

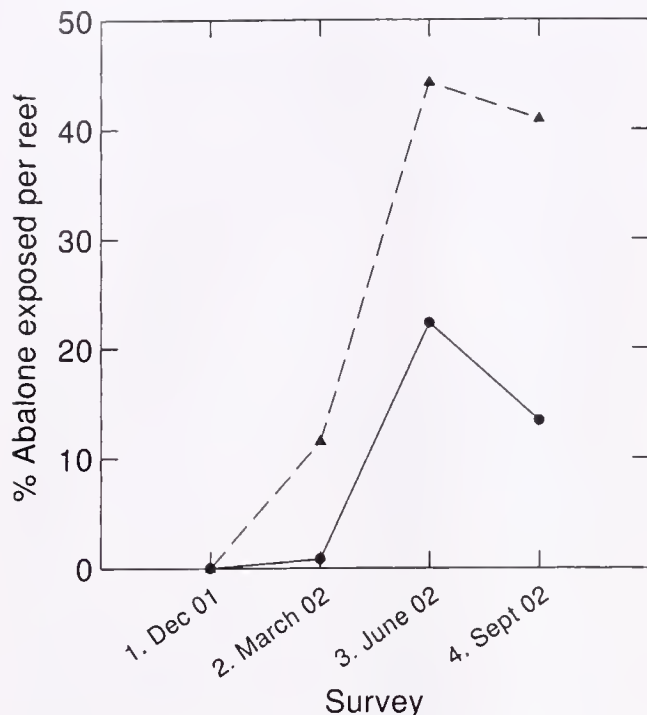


Figure 3. Plot of the proportion of *H. laevis* found on the edge of reefs at each survey. Data from all sites and reefs are pooled. Circles (solid line) = High density; Triangles (dashed line) = Low density.

juveniles of the same age at West Island (a nearby site with similar characteristics). Shepherd (1988) showed that wild juveniles consistently reached 25–29 mm in their first 18 mo in a study of 5 separate cohorts at West Island, whereas Shepherd and Hearn (1983) recorded growth rates of up to 39 mm in the first year at the same location. The experimental reefs were assembled from natural substrates complete with predators and interspecific competitors. Treatment densities, in terms of the number of abalone per area of suitable habitat, may also be compared with densities of wild adult *H. laevis* found by Dixon and Day. The growth rates we observed in the field for our study (~40 mm per year) were comparable to those seen in wild juveniles of a similar size (Shepherd & Hearn 1983, 33 mm per year; Shepherd 1992, 43.4 mm per year). The mortality rates recorded in our experiment (see Dixon et al. 2005) were also similar to those found in wild juvenile populations (Shepherd 1998). With all of these considerations, we are confident that our results provide valuable and relevant information for wild stocks.

The size of abalone, in terms of length or weight, seems to be the most important determinant of maturation in *H. laevis*. Considering the known age of all abalone released into the field (30 mo in September 2002), we can conclude that, in a maturing population, larger abalone would become reproductive one or more years before smaller abalone of the same age. This is an important finding in the light of other studies that suggest maturity in wild abalone is genetically determined on reaching a certain age (Shepherd & Laws 1974, Prince et al. 1988, Nash 1990, Shepherd et al. 1991, Nash 1992). Because larger abalone were the faster growing individuals over the year, our results strongly suggest that there is significant plasticity in the age at which *H. laevis* become mature and that this age varies with growth rate.

Abalone in this experiment were of a similar size when re-

leased, so differences in the growth rate of individuals are responsible for whether these abalone matured in 2002 and their degree of gonad development. Significantly, juvenile somatic growth during the first 3 mo of the study was most important in determining both final length and the amount of gonad developed by September, whereas growth in subsequent periods was not strongly correlated to the VGI. The strong influence of initial growth rate on subsequent gonad development might be attributable to stress induced during seeding (Schiel 1993), such that less stressed individuals that grew well immediately following release had a significant advantage over other abalone. It is possible, therefore, that growth and the timing of maturation in abalone will be sensitive to other environmental stresses.

Another intriguing possibility is that seasonal growth (i.e. in Spring) when food is more abundant is especially important for maturing abalone. Another hypothesis is that individual juvenile abalone may have differing inherent growth rate potentials by virtue of some aspect of body condition. We were unable to separate these hypotheses in our experiment, but suggest that stress is likely the dominating influence.

It remains to be determined whether early growth performance in a natural cohort of juveniles would be a strong determinant of the timing of maturation. It is believed, however, that nutritive differences very early in life will shape growth and maturity patterns as animals grow (Stearns 1992). Indeed, early growth in abalone is often associated with later growth performances in the hatchery (Huchette 2003).

Abalone at high-density grew more slowly than those at low density, as reported in aquaculture studies (Mgaya & Mercer 1995, Capinpin et al. 1999, Huchette et al. 2003) and in the field (Dixon & Day 2004). Density also seems to affect the timing and degree of gonad development in *H. laevis*. Abalone at high-density seemed to initiate gonad development at smaller sizes than those at low-density (Fig. 2). This tendency amongst slow growing populations of organisms is well documented in life history theory (Stearns & Koella 1986) but this study constitutes direct evidence of this in abalone.

The finding that, overall, a higher percentage of abalone from low density reefs were mature is clearly an indirect effect, related to the size of abalone at low density. That is, abalone at low density grew faster. The indirect effect of density on maturation is important because in years of low recruitment to an abalone population, compensatory increases in the growth of young individuals are likely (if levels of interspecific competition are the same) to increase the average individual egg production from that cohort.

It has been suggested that abalone populations with fast growth tend to mature at a younger age than slower growing populations (Wells & Mulvey 1995, Shepherd & Triantafyllou 1997). Our results correspond with this since large, faster growing *H. laevis* began to show signs of maturity at least 1 y before slower growing individuals. This suggests that the timing of maturation in abalone is a plastic trait in response to growth rate and that maturation is not genetically determined to commence at a given age. Empirical evidence for this plasticity was provided by a study on *H. iris* by McShane and Naylor (1995a). Based on length-at-age data, McShane and Naylor found that abalone that were exposed to higher flow rates (and hence more food) near headlands tended to grow faster, and mature earlier, than those in sheltered bays. Our study is the first to demonstrate plasticity in the timing of maturation within a local abalone population and our results are consistent with findings in other marine invertebrates (Thompson 1979, Franz 1996) and in fish (Stearns & Crandall 1984, Reznick 1990).

Anecdotal evidence from aquaculture studies suggests that small *H. laevigata* (50–60 mm) can be successfully induced to spawn in the hatchery (John Hall, pers. comm.). This may represent a size limit in *H. laevigata*, below which individuals are physiologically incapable of spawning. Histologic examination of gonad tissue from Cape Jervis abalone (all of which were >60 mm) suggested that larger individuals with more gonad would be capable of spawning but, because of their relatively small size, gamete production would be low.

If these abalone gain one or more extra spawning seasons due to their earlier maturation, this could be significant in the management of the fishery because the reproductive output of a cohort will vary considerably as individuals approach sexual maturity. It is possible that larger abalone at Cape Jervis actually spawned in 2002. The fact that these abalone had already developed gonads at 30 mo, and were also the fastest growing individuals in the cohort, might allow them to produce larger gonads in future spawning seasons than other abalone. It is also possible that abalone with faster early growth may reach a higher asymptotic length (and hence higher reproductive capacity, Stearns 1992), but our data cannot test this hypothesis. In either case, abalone that mature earlier have a substantial reproductive advantage over slower growing abalone that have remained small (Roff 2002).

Prince et al. (1988) and Nash (1992) suggested that the commencement of maturation and end of exclusively somatic growth also coincided with the emergence of *H. rubra* to less cryptic positions on rocks, characteristic of adult habitat. The results for *H. laevigata* at Cape Jervis correspond with this because many of the abalone that developed gonad were found to be on the edge of plots in June where they sat on rocks bordered by sand and seagrass. This position would give abalone better access to drift algae (Shepherd 1973) and is characteristic of adult *H. laevigata* habitat. It is important to note that in our experiment, densities of adult *H. laevigata* were low on experimental reefs such that adult habitat was largely vacant. In populations where adult densities are higher, juveniles may face stronger competition for positions on the edge of reefs.

The emergence of large numbers of abalone to the edge of plots between March and June seems to precede the development of gonad tissue, which is believed to take place between June and September (Shepherd & Laws 1974, Shepherd & Hearn 1983). This may reflect a delay between when more food becomes available and the energetic allocation of resources to growth or reproduction (Day & Fleming 1992, Donovan & Carefoot 1998).

Abalone that were on the edge of reefs in June would have access to more abundant food and were subsequently likely to have developed gonad. This suggests that the amount or quality of food available to juvenile abalone may be important in the initiation of gonad development. Nutrition is known to play an important role in the timing of maturation and is strongly correlated with growth rate in other invertebrates (Lucas & Lawes 1998, Boyd et al. 1986) and in fish (Reznick 1990). Thus differences in growth rate and maturation between high and low-density abalone presumably reflect the more basic difference of increased nutrition.

Across many taxa (including humans), poorly nourished individuals mature much later than well-fed individuals (Stearns & Koella 1986). Our study shows that this is also true for abalone, because animals with greater access to food resources (e.g. those at lower experimental density and/or residing on the edge of reefs) were likely to mature in 2002 whereas abalone with poor access to food would likely not mature for another year.

The age at first maturity in *H. laevigata* seems to be a highly plastic trait in response to different growth rates and/or nutrition levels. The results of our study indicate that the onset of maturation is not fixed at a certain age within a population but can be advanced under conditions of fast growth.

ACKNOWLEDGMENTS

The authors thank all those who helped with survey diving and collection, especially James Brooks. We also thank Dr. Scoresby Shepherd and two anonymous reviewers for their helpful comments on earlier drafts of the manuscript.

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ABALONE RESOURCE DECLINE AND A RECOVERY ATTEMPT IN CHIBA PREFECTURE, JAPAN

KANAKO UCHINO,* TOSIATU SIMIZU, TANE0 TANAKA, AND TERUKAZU SHIBATA

Chiba Prefectural Government, Fisheries Research Center

ABSTRACT Landings of abalone (*H. discus*, *H. gigantea*, and *H. madaka*) in Chiba Prefecture, Japan exceeded 700 t in 1966 and fluctuated between 600–800 t until 1978. The landings started to decline in 1979 and reduced to less than 100 t in 1997. Although landing of *H. discus* has been slightly increased since 2000, that of *H. gigantea* abalone is still decreasing. We analyzed the data of investigations on decline in abalone landings and have come up with “environment theory”, “over-fishing theory” and “disease theory.” In the environment theory various factors were considered, such as biomass decrease of purple sea urchin; which is used as a shelter by juvenile abalone, sedimentation flux increase, withering of seashore (Isokayake), oceanic dynamic change, predation damage, coastline development, and endocrine disruptors, of which the sedimentation increase was thought to be the most influential factor. In the over-fishing theory, decrease in the number of reproductive adults due to reinforcement of the fishing intensity was considered to reduce the fertilization success and caused the decline in recruitment. The disease theory was found to be not applicable because virus infection of seeds has not been reported in Chiba Prefecture. To solve the problems related to decrease in adult population number, various research approaches are possible: development of techniques to create suitable reproduction habitat, long term observation of reproductive conditions in a perpetual observation area, improvement of seed transportation and release methods, and development of fishing ground construction technology. We have been conducting research on the effects of leaving the fishing ground fallow for 5 y, and we are also investigating the amount of resources in different developmental stages. The present study summarizes the abalone landing decline factors in Chiba Prefecture and describes the present status of our research.

KEY WORDS: fishing intensity, recovery, abalone, *Haliotis*

INTRODUCTION

In order for Chiba Prefecture to work on the investigation and the measure against production increase of abalone resources, the “abalone resource recovery project,” which consists of administration, test research, and fisheries extension section was established in 1998. To clarify the causes of resource reduction, environmental theory; over-fishing theory; and disease theory were built and examined (Simizu & Tanaka 2001).

ENVIRONMENTAL THEORY

Biomass Decrease of Purple Sea Urchin

Although juveniles are considered to hide under the prickly of purple sea urchin to escape from predator (Kojima 1974, Kojima 1981, Tanaka & Tanaka 1982), purple sea urchin is not necessarily distributed thoroughly in Chiba Prefecture (Anonymous 1980, Anonymous 1981), and abalone juveniles were observed to move out from underneath the spines of purple sea urchin in approach of eight armed starfishes (*Coscinasterias acutispina*). Therefore, decrease of purple sea urchin may not be a major reduction factor (Tanaka 2001).

Sedimentation Flux Increase

There is a report that sedimentation flux influences survival of abalone larvae and juvenile (Yamada & Kamimura 1973, Sakamoto 1979, Sakamoto & Nanba 1980). Growth and survival rate of early juveniles are known to be better in filtered sea water.

Withering of Seashore

Seashore withering is reported to deteriorate food conditions of abalone, causing malnutrition and decline in abalone resources

(Kawajiri et al. 1981, Tanaka & Tanaka 1986). There is a report that production of food seaweed influences survival of *H. discus hannai* (Sakai 1962). However, in the Hamanamekawa and Kawaguchi area where the abalone fishery is performed, flora and the amount of sea oak and sea trumpet, which are the food items of abalone, did not differ between 1978 and 1998 (Tanaka & Simizu 2000).

Ocean Dynamic Change

In *H. discus hannai*, water temperature decrease by cold Oyashio current is one of causes of stock size decline (Shibuya 1984). The Chiba coast is under influence of warm Kuroshio cur-

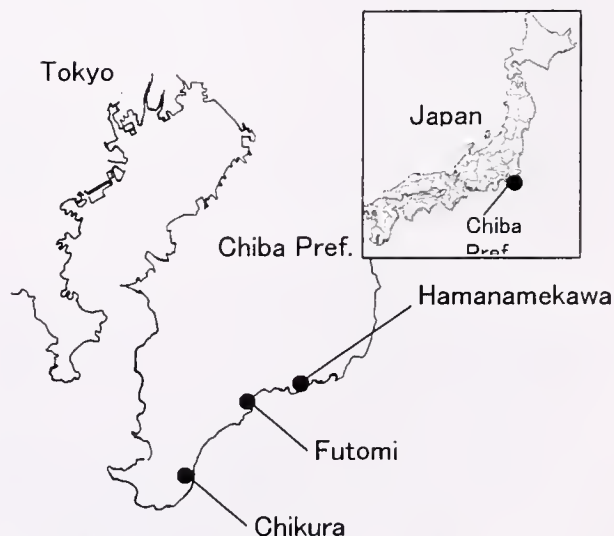


Figure 1. Map of Chiba Prefecture with abalone research location in this study.

*Corresponding author. E-mail: k.uchino@mc.pref.chiba.jp

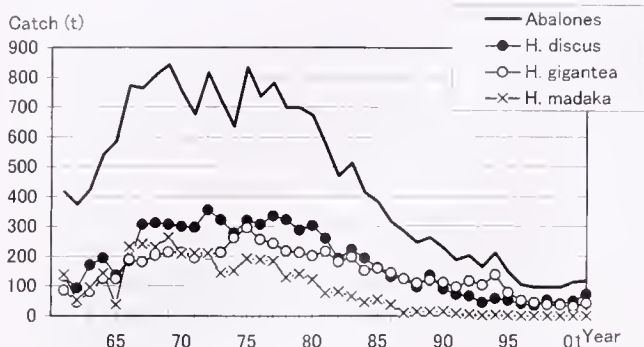


Figure 2. Trend of abalone catches from 1960 to 2001 in Chiba Prefecture. The catches started to decline in 1979 and reduced to less than 100 t in 1997. Although catches of *H. discus* has been slightly increased since 2000, that of *H. gigantea* abalone is still decreasing. *H. diversicolor aquatilis* is included in total catch.

rent. Although winter water temperature has been rising slightly since 1986, abalone catch has begun to decrease since 1980 in Chikura areas (Tanaka & Simizu 2000). Thus, on the Chiba coast, water temperature may not be a reduction factor because ocean dynamic change and resource change do not synchronize.

Predation Damage

Octopus, crabs, star fish, snails, and fishes are known predators of abalone (Kojima 1981, Yamakawa 1990, Seimura et al. 1993, Anonymous 1976). However, detailed data of predation damage of abalone are not available. No correlation is found between landings of octopus and abalone (Simizu & Tanaka 2001). This may indicate minor importance of predation on abalone stock, but further studies are required.

Coastline Development

There has been no large scale coastal development in Chiba. Although there supposed to be some local fishery loss by the outflow of earth and sand, damages has been relatively minor.

Endocrine Disruptors

Abnormalities in the gonads of abalone, which are known to be caused by endocrine disruptors, may reduce reproductive activity of abalone (Horiguchi & Shiraishi 1999). However, histologic abnormalities have not been observed in *H. discus* and a *H. gigantea* from Kawaguchi area (Simizu & Tanaka 2001).

OVER-FISHING THEORY

There are reports that describe abalone resource decline due to reinforcement of fishing intensity (Anonymous, 1984, Nonaka 1987, Tachiyama et al. 1998, Horii 1998). In Chiba Prefecture, the possibility that introducing wet suit to diving fishery and/or other elements caused the over-fishing cannot be denied.

The habitat density of 1-y-old abalone was remarkably decreased in 1989 as compared with 1978 (Tanaka & Simizu 2000), indicating the decrease of new recruitment. In Australian greenlip abalone (*Haliotis laevis*), high density of broodstock is known to be indispensable for fertilization success (Babcock & Keesing 1999). Decrease in the number of reproductive adults due to rein-

forcement of the fishing intensity was considered to reduce fertilization success and caused the decline in recruitment.

DISEASE THEORY

Although wild abalone may die of virus infection passed on by infected artificial seeds, no virus infection has been reported in the process of the seed production in Chiba Prefecture. Therefore, virus infection does not seem to be major reduction factor of abalone resources.

In summary, the main causes of the abalone resource reduction in Chiba Prefecture seem to be related to over-fishing and we took a measure against recovery based on this conclusion.

Solution for Resource Recovery

Improvement of Abalone Seed Release Method

The recovery rate of the released seeds in Chiba Prefecture is estimated to be 8.8% (Sakamoto et al. 1986). By improving the release method, the survival rate, and therefore recovery rate, may be increased.

The survival rate of abalone seeds can be maximized by the use of a release basket (Fig. 3) (Nishima & Ito 1989, Tanaka & Sakamoto 2001). To date, divers have released abalone by hand in Chiba Prefecture. However handling can damage the seeds. The introduction of a release basket can make transporting and releasing of seeds more efficient. A study of this method was performed to test its effects. The release basket, which is used for *H. discus hannai* release in Iwate Prefecture (Anonymous, 1990), can accommodate about 1500 individuals of seeds (~3-cm SL). The examination was carried out in a developed area with about 200 concrete boards (60 cm × 60 cm) in Hamanamekawa area in November 2001.

Effect of the release basket was examined. The use of the release basket increased the recapture rate of *H. gigantea*, whereas the effects were not clear in *H. discus* (Table 1). To mitigate the predator damage of released seeds, water temperature should be low. Use of the release basket, which can make it possible to carry out the release without diving in the winter, may be effective.

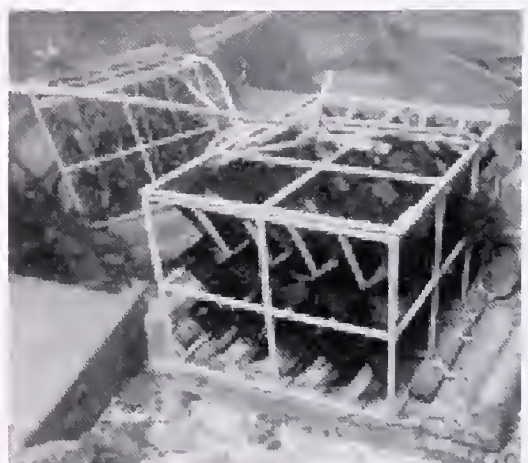


Figure 3. Release basket, a concrete slab with slats to fit PVC panels to which abalone juveniles are attached.

TABLE 1.

Comparison of recovery rates between two release basket and diving, in *H. discus* and *H. gigantea*.

Method	Species	At Release Nov. 7, 2001			At Recapture Dec. 4, 2001			
		N	Shell Length (mean \pm SD mm)	Weight (g)	N	Recapture/rate (%)	Shell Length (mean \pm SD mm)	Weight (g)
Release basket	H. discus	1000	29.8 \pm 1.4	3.7 \pm 0.5	332	33.2	30.4 \pm 1.7	3.6 \pm 0.8
Diving		996	29.8 \pm 1.4	3.7 \pm 0.5	354	35.5	30.2 \pm 1.6	3.7 \pm 0.6
Release basket	H. gigantea	990	27.8 \pm 1.4	2.8 \pm 0.5	622	62.8	28.9 \pm 1.6	2.8 \pm 0.5
Diving		959	27.8 \pm 1.4	2.8 \pm 0.5	486	50.7	29.0 \pm 1.8	3.0 \pm 0.6

Development of Suitable Reproduction Habitat

We have been conducting research on the effects of catching abalone every 3 y. One research area 1,000 m², and was developed with about 1,500 sheets of the concrete plates (80 \times 60 \times 10 cm). About 20,000 *H. discus* seeds were released in one area, and the recovery rate was examined after 3 y. Three sets of areas established and "catch rotation during 3 years" is carried out. The amount of recoveries been 300–400 kg per one area on average (Table 2), 700 kg maximum, which is about 2,000 individuals. In an average fishery ground, the benefit of seeding greatly exceeds the cost. This type of catch rotation fishery can expect not only increased recovery of released abalone but it is also functions in the maintenance of high parents density.

Early Ecology of Abalones in Chiba Coast

To attain suitable and effective resource control and enhancement, investigation of resource change mechanisms is indispensable.

The occurrence of planktonic larvae Tidal the flow was investigated, and abalone larvae were collected using the Kitahara plankton net in Fromi area in 1979 and 1980. The spawning season of abalone was considered to start in late October and end in February, and it became clear that spawning is induced by low atmospheric pressure and rapid water temperature changes. Eggs and larvae were found to be accumulated in the whirlpool region (Tanaka & Ishida 1986). To compare with the past conditions, larvae were collected by the Kitahara plankton net in 2001 and 2002 (Table 3). The densities of *H. discus* and a *H. gigantea* were

0.4 ind./m⁻² and 0.5 ind./m⁻² respectively in 1987 and were 0.01 ind./m⁻² and 0.02 ind./m⁻² in 1998. Despite the fact that there were larger amount of resources 20 y ago, the numbers of larvae collections of the larvae in 2001 and 1980 were in the same level. The occurrence of larvae is a key factor in understanding abalone stocks, yet it is the most elusive. It is necessary to carry out studies on methodology.

The occurrence of postlarvae Occurrence of postlarvae of abalone has been investigated since 1977. We collect natural stones and concrete boards (20 \times 20 \times 2 cm) to extract the juveniles at least once a week in the Kawaguchi area from late October to December, which is the breeding season of abalone. The number of annual accumulation of postlarvae (m⁻²) was the highest in 1977 (i.e., 277) and gradually decreased thereafter to 0–7 in the 1990s (Tanaka & Kasai 2000). This trend seems to be in parallel with the trend of abalone stock abundance: systematic accumulation of knowledge of ecology and methodology is necessary.

CONCLUSION

For abalone stock enhancement, improvement in the survival and recovery rates after the release is raised as a short-term objective, and it is necessary to steadily advance and spread the technologies introduced in this study. Moreover, because the current status of fisheries will not fall to growth-over-fishing if the present legal size (i.e., 12 cm) of capture is kept (Simizu 2000), appropriate measures such as enlightenment on fisheries association consciousness, poaching prevention and shell length restric-

TABLE 2.

Landings of abalone in rotation fisheries grounds.

Area	Kawaguchi		Hiraiso		Senda		Okawa		Shiramazu	
	Catch (kg)	Rate* (%)	Catch (kg)	Rate* (%)	Catch (kg)	Rate* (%)	Catch (kg)	Rate* (%)	Catch (kg)	Rate* (%)
P1	517	74.4	269	89.2	245	85.1	448	71	105	46.4
P2	185	76.7	738	92.9	170	55.8	540	93.1	266	62.8
P3	517	70.9	603	89.7	176	69.6	179	68.6	245	69.5
K1	153	86.6	588	86.9	376	88.7	306	85.4	270	95.1
K2	136	62.1	686	97.2	437	91.4	434	92.7	309	93.2
K3	585	91.7	414	90.5	479	97.5	398	89.4	296	88.4
mean	349	77.1	550	91.1	314	81.4	384	83.4	249	75.9
The amount of recoveries	269		501		255		320		189	

Fishing was annually rotated in two groups: P1–P3 and K1–K3.

* Rate: ratio of released abalone in the landings

TABLE 3.

Occurrence density of abalone larvae in Futomi area, Chiba Pref.

Year	Number of Investigation	Mean Density (ind./m ³)	
		Veliger	Egg and Trochophore
1979	10	27.8	34.7
1980	9	1.3	19.1
2001	10	1.0	—
2002	7	0.4	—

tion observance, and an improvement of fisheries management system, are indispensable.

At the same time as a mid and long-term objective, the early ecology of abalones and a resource change mechanism need to be elucidated towards the increase in natural juvenile production. Accumulation of the systematic knowledge is desirable.

ACKNOWLEDGMENTS

The authors thank Dr. Toyomitsu Horii and Dr. Satoshi Watanabe, National Research Institute of Fisheries Science, for their critical reading of this manuscript.

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GROWTH RESPONSES IN EMERGENT GREENLIP ABALONE TO DENSITY REDUCTIONS AND TRANSLOCATIONS

CAMERON D. DIXON AND ROBERT W. DAY

Zoology Department, The University of Melbourne, Victoria

ABSTRACT Growth of “stunted” greenlip abalone in areas with low maximum sizes was enhanced over 6 mo by reducing their natural density and by translocation to habitats supporting faster growing abalone. Density reductions significantly increased growth relative to controls, apparently without altering the asymptotic length. Stunted abalone showed a consistent and similar pattern of enhanced growth when translocated to two sites where abalone characteristically grow faster and to larger sizes. When compared with slow growth control abalone, the response of translocated abalone varied with initial length in the same manner as in the experiment where density was reduced. When compared with fast growth controls, translocated abalone had similar trends in growth increment versus size, yet all size categories grew consistently less. Statistical comparisons cannot be made between density-reduced and translocated abalone because abalone in the density reduction experiments were tagged *in situ*, whereas translocated abalone were tagged aboard a research vessel. The fact that reduced density and better quality habitat positively influence growth patterns of greenlip abalone, producing the same short-term response, suggests that food availability may limit the growth of stunted populations. The asymptotic length, which appeared unaffected in both experiments, may be determined by long-term conditions, or perhaps by conditions during the onset of maturity.

KEY WORDS: abalone, *Haliotis laevis*, density-dependence, growth, density-reduction, translocation

INTRODUCTION

An understanding of density dependent processes is essential to fisheries management, particularly compensatory effects that can offset natural or fishing-induced reductions in biomass (Rose et al. 2001). Several studies have reported interspecific competition that resulted in density dependent changes in growth rate (Kamermans et al. 1992, Brazeiro & Defeo 1999, Barki et al. 2001, Talman & Keough 2001, Vromant et al. 2002), but experimental manipulations of density to determine effects of intraspecific competition in the wild are often logistically difficult, particularly for subtidal species.

Stoner (1989) showed that growth of the queen conch, *Strombus gigas*, was significantly different at densities two and four times those observed in the wild and he suggested that this result was food related. Marshall and Keough (1994) also provided evidence of density-limited growth for the limpet, *Cellana tramoserica*. Abalone, like conch and limpets, can often be found in high-density aggregations (Shepherd 1986, McShane 1995, Officer et al. 2001), and because they feed on drift algae it is reasonable to postulate that abalone density, as a result of intraspecific competition for food, may affect growth.

Most abalone research investigating density dependent growth has been restricted to aquaculture experiments (Hunt et al. 1995, Mgaya & Mercer 1995, Capinpin Jr. et al. 1999, Huchette et al. 2003), and in each case growth was negatively correlated with density. In contrast to these results, McShane and Naylor (1995a) reported density independent growth of *Haliotis iris* from field experiments in New Zealand. In their experiment McShane and Naylor artificially increased densities within enclosures, using natural densities for the controls. They acknowledged that a more realistic experiment to test density effects would be achieved with a reduction, rather than increase, in density. “Stunted” populations of abalone (McShane et al. 1994, Wells & Mulvey 1995), where few individuals will ever grow to the legal minimum length and therefore most will remain unfinished, provide a unique opportunity to conduct such manipulative experiments in a population at natural density.

Translocation of abalone to habitats with greater food abundance or quality has been shown to increase growth rate. Emmett and Jamieson (1989) transplanted stunted *H. kamschatkana* to better quality habitats and obtained growth rates twice those of control populations. McShane and Naylor (1995b) also observed significant increases in growth rate when *H. iris* were translocated from bay to headland habitats. A review by Day and Fleming (1992) concluded that variation in abalone growth was primarily due to the quality and quantity of algal food available. Assuming that density affects food availability per individual, a reduction in density could be expected to invoke a similar growth response as translocation of abalone to better quality habitats.

Our experiment, conducted over a 6-mo period, studied the effect of density reductions at three sites with stunted abalone at Tiparra reef, South Australia. Abalone removed from two of these sites were translocated to locations with fast growing abalone, and controls of both stunted abalone and fast growing abalone were tagged to determine the effect of changing habitat on growth.

METHODS

During March and April 2002, 2,940 greenlip abalone were tagged at five sites on Tiparra Reef, South Australia (Fig. 1), using rivet tags inserted into a respiratory pore of the shell (Prince 1991). Tagged abalone (1,873) were recaptured and measured for shell growth during September 2002.

The experiment to determine the effect of density on shell growth was established at three sites where abalone grew to small maximum lengths. We assumed that growth at these sites would be slow, and the results confirm this. These sites were within a kilometer of each other, separated by areas of unsuitable habitat. Within each site two adjacent areas were marked out and measured, and abalone were tagged *in situ* in each area. We did not anticipate extensive movements during these experiments because Shepherd (1973) observed that movements were minimal over long periods of time at a site near to the Tiparra Lighthouse. We therefore established the control areas only 10 m from treatment areas to minimize differences between them. In control areas all emergent abalone were tagged, and in treatment areas every third



Figure 1. Location of sites at Tiparra reef, South Australia.

emergent abalone encountered was tagged, during systematic searches of the areas. After tagging was completed all untagged emergent abalone in treatment areas were removed. The size of density-reduced areas was approximately three times larger than control areas to ensure similar numbers of tagged individuals after thinning.

Recapture surveys also involved systematic searches of each area, but when aggregations were encountered, the numbers of tagged and untagged abalone were counted. At each site the original density was estimated using the number tagged in March, and the final density in September was calculated from both the number recaptured and the proportion of tagged to untagged abalone in aggregations during the recapture surveys. This latter density estimate assumed that the proportion of tagged individuals within the aggregations measured reflects the proportion of tagged to untagged individuals in the remainder of the area. An aggregation was defined as a group of abalone with no more than 150 cm between two individual abalone, a key distance for fertilization success of *H. laevigata* (Babcock & Keesing 1999). A high proportion of abalone were in aggregations at all sites. Only abalone found in their area of origin were included in the abundance estimation.

Each site varied in degree of aggregation as well as habitat. The "Lighthouse" site was at 3-m depth, with smaller aggregations of abalone that were relatively evenly distributed on continuous limestone habitat. The "Aggregation" site consisted of small to large aggregations of tightly clustered abalone at 6-m depth. Aggregations were found on patches of flat limestone reef among seagrass. The third site, "Sand Gutters," also at 6-m depth, was established among parallel gutters of limestone reef in between raised ridges of sand. The dominant seagrasses at each site were *Posidonia* spp. but there were small patches of *Amphibolus antarctica* and a variety of macroalgae. Although the sites varied greatly in habitat, few abalone in each area reached the legal minimum size (130 mm), and their densities were consistently high prior to thinning. Commercial abalone divers reported that these areas were rarely fished because large abalone were always scarce.

For the translocation experiment two sites where abalone grew to large sizes were established approximately 1 km from the "stunted" sites. Both these sites contained abalone at densities and sizes typical of productive commercial fishing grounds, with an average size approximately 15 mm larger than the slow growth sites. Commercial divers had not fished at either fast growth site for at least 3 y. These sites were surrounded by luxuriant stands of *Posidonia* spp. and *Amphibolus antarctica*, with a large loading of epiphytic algae (suitable greenlip food) during surveys in March and September.

Abalone were removed from each fast growth site and a proportion was tagged on the boat and returned to the bottom as fast growth controls. The remainder were removed from the site to allow the translocation of tagged abalone from slow growth sites while maintaining the original density of abalone at the site. Densities of abalone at the two fast growth sites approximated those of slow growth sites. Abalone removed during thinning of the areas at the Lighthouse and Aggregation sites were tagged at the surface and translocated to Fast Growth Sites 1 and 2 respectively. Surface exposure times were similar for all treatments. At the fast growth sites similar numbers of treatments and control abalone were mixed within the same habitat. A second control area was established at each of the Lighthouse and Aggregation sites. Abalone were removed from the bottom and tagged at the surface before being returned, to replicate the method of tagging for transplants and at the fast growth sites.

Abalone were recaptured and measured from each area in September. Incremental growth was standardized to 180 days and regressed against the release length. Because growth increment is expected to vary with initial size, size was included as a covariate, and size-specific responses to treatments were expected. Because Shepherd and Hearn (1983) found growth differences between sexes at one of the sites they investigated, initial ANCOVAs for each site and treatment were set up to test whether growth varied between the sexes. Because we did not detect significant differences between sexes ($P > 0.05$), the data were pooled for subsequent analyses. Wells and Mulvey (1995) also found no significant differences in growth rate between sexes for *Haliotis laevigata* in Western Australia.

ANCOVA assumes the covariate is similarly distributed between treatments (Quinn & Keough 2002). In the thinning experiment only data from the Aggregation site contained a different range of sizes for the two treatments. Trimming the data to contain only individuals greater than 90 mm made no difference to the significance of the test and therefore the data from the thinning experiment were not trimmed. In the analysis of the translocation experiment the data were trimmed to include only abalone between 80 mm and 160 mm, to ensure all treatments extended over the same range of sizes. We checked whether the relation of growth increment to initial length was linear by plotting the mean increments of 10-mm size classes. Confidence intervals were calculated for asymptotic lengths (the X axis intercept) from each regression using formulae for X axis intervals in Snedecor and Cochran (1967).

RESULTS

At the Aggregation and Sand Gutters sites the densities remained similar over the 6-mo period; at the Aggregation site density was reduced from 33% of original density after thinning to 29% in September, and at the Sand Gutters site it increased slightly

TABLE 1.

Estimation of abundance from the proportion of tagged to untagged individuals encountered within sites at Tiparra reef.

Site	Treatment	March Abundance	Number Tagged	% Abundance Post-thinning	Total Tags Recaptured	% in Aggregations with Tags	Estimated September Abundance	% of March Abundance
Lighthouse	Thinned	603	201	33%	74	19%	389	65%
	Control	246	246	100%	99	51%	194	79%
Aggregation	Thinned	810	270	33%	142	61%	233	29%
	Control	269	269	100%	215	81%	265	99%
Sand Gutters	Thinned	354	118	33%	85	67%	127	36%
	Control	135	135	100%	97	93%	104	77%

from 33% to 36% (Table 1). The Lighthouse site showed an increase in density consistent with some degree of immigration, from 33% after thinning to 65% after 6 mo (Table 1). The proportion of tagged to untagged individuals reduced from 100% to 19% 6 mo later, and only 37% of all tagged abalone were recaptured within the area. Nevertheless, the average density (the mean of 33% after thinning and 65% after 6 mo) is approximately half of the original abundance. Control area densities remained about the same at the Aggregation site but reduced slightly at the Lighthouse and Sand Gutters sites. At these two sites the average decrease in density was only 10% after 6 mo.

Analysis of covariance was performed using data from each of the three sites. The treatment by length interactions were significant; that is the slope of growth rate versus length differed between the control and thinned areas, at each site (Table 2). These tests produce conclusions that relate to each site and might be due to chance differences between the two areas. Sites were then used as replicates to test the effects of density reduction. The treatment by length interaction was tested against the 3-factor interaction because sites were a random factor. This test was significant ($F = 67.969$ $df = 1, 2$; $P < 0.05$), and we conclude that thinning changes the relation between size and growth.

At all sites smaller abalone showed more rapid shell growth in response to thinning (Fig. 2). The size-specific rates of growth at the Aggregation and Sand Gutter sites were similar when compared between control areas and between thinned areas. At the Lighthouse site however, growth rates were much higher, such that size-specific growth at the Lighthouse control was similar to growth in the thinned areas at the other two sites. Nevertheless, the magnitude of the difference between treatments and controls at any given length was very similar at all three sites. The asymptotic lengths of the abalone (X axis intercept) were similar at all sites

and treatments, except for the controls at the Aggregation site (Table 3).

The translocation experiment data were analyzed separately for each site as fast growth control versus translocated and slow growth control versus translocated (Table 4). In analyses of covariance for the translocated versus slow growth controls the treatment by length interaction was highly significant at the Aggregation site (Table 4) but not at the Lighthouse site, probably because there were only 46 recaptures at the Lighthouse slow growth control site. There was, however, a highly significant difference between thinning treatments at the Lighthouse site (Table 4). For translocated versus fast growth controls there was no significant treatment by length interactions, so this interaction term was omitted from the model. In both cases there were highly significant differences between treatments (Table 4). No analysis using sites as replicates was performed because there were only two sites in the experiment.

For translocated abalone from both the Lighthouse and Aggregation sites, the patterns of growth were consistent (Fig. 3). Translocated abalone responded in a similar manner to thinned abalone when compared with slow growth controls, with smaller abalone showing more rapid growth in response to the change of habitat, whereas there were no significant differences in asymptotic length (Table 5). When compared with fast growth controls, translocated abalone grew at a consistently slower rate across all lengths (Fig. 3), resulting in significant differences in asymptotic length at both sites (Table 5). Once again the magnitude of the response in both cases was similar, despite differences in growth rates between the sites.

DISCUSSION

The growth differences between thinned and control areas were consistent at all three sites despite differences in the magnitude of the density reduction. There were indications of substantial immigration leading to increasing density within thinned areas after 6 mo at the Lighthouse, but not at other sites. There was minimal cryptic habitat and therefore emergence was not likely to have contributed to the increase in density. Presumably, abalone migrated into the area because of the relatively greater abundance of food. At both the Lighthouse and Sand Gutters sites a small reduction in the abalone density within control areas, only 10 m away, was observed. Officer et al. (2001) obtained identical patterns of movement and reaggregation during density reduction experiments of blacklip abalone, and they suggested that the reduction in density within control areas was an indirect consequence of redistribution of individuals into the thinned areas. Be-

TABLE 2.

Tests of differences between slopes in thinned versus control areas, using analysis of covariance at each site.

Site	Source	DF	Mean Squares	F-ratio	P-values
Aggregation	Treatment \times Length	1	55.891	7.945	0.007
	Error	351	7.457		
Gutters	Treatment \times Length	1	36.681	6.032	0.015
	Error	175	6.081		
Lighthouse	Treatment \times Length	1	73.208	8.227	0.005
	Error	169	8.899		

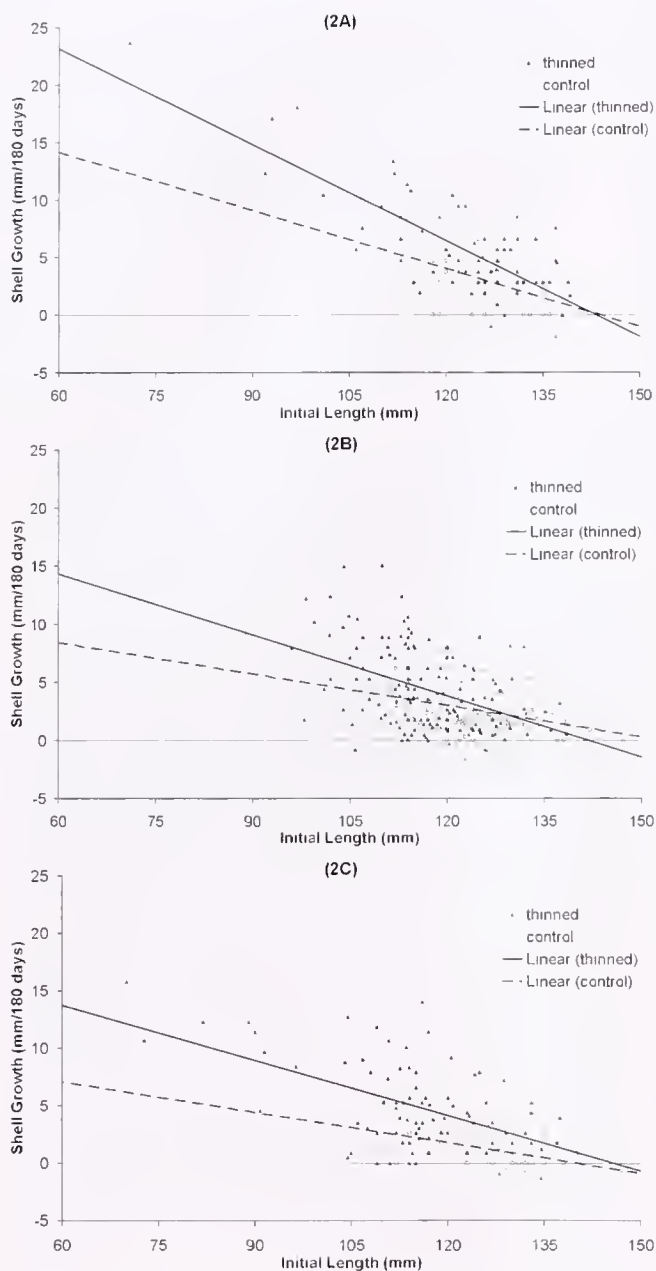


Figure 2. Growth increment versus initial length for control and thinned areas at the (A) Lighthouse site, (B) Aggregation site, and (C) Sand Gutters site.

cause of the closeness between control and treatment areas in our experiment, decreases in control density may also have been a consequence of movement into thinned areas, but we did not find any tagged controls in thinned areas.

We expect that tag loss was minimal over our 6-mo experiment, but note that because we estimated final density from the number tagged and the proportion of tagged to untagged during the recapture dives, tag loss would not have affected our density estimates. Mortality of adult abalone over 6 mo would be expected to be very low. The average density over 6 mo at the Lighthouse site was 50% of the original density in the thinned areas and 90% of original density in control areas, effectively equating to a 40% reduction. The average density reduction at the Aggregation and Lighthouse sites were 70% and 60% respectively. Despite the extent of

movements at the Lighthouse site, an average density reduction of only 40% was sufficient to establish significant differences in growth rate between thinned and control areas, comparable with the other two sites.

The consistent differences observed led to the overall test result: that density reduction led to an increased slope of the increment versus length regression. The slope of these regressions reflects the Von Bertalanffy growth parameter k , and the X intercept approximates L_{∞} (Day & Fleming 1992). It is clear that for slow growing populations of greenlip abalone, density reduction increases k , but in general it seems not to change L_{∞} much, at least in the short term. This response may reflect the preference of juvenile abalone to direct extra resources into shell growth, whereas larger, older individuals may be placing more of these resources into maintenance and reproduction, a trade-off common to many species (Stearns 1992). L_{∞} was significantly greater in the control area at the Aggregation site than at all other controls and treatments. This difference may be a consequence of the relatively small number of large abalone in the thinned treatment at this site.

At the fast growth sites, a greater food supply was clearly evident during both March and September, because areas of adjacent seagrass had much larger epiphyte loads compared with slow growth sites. These differences in food availability presumably led to the differences in growth and asymptotic length between slow growth and fast growth controls.

We hypothesized that individuals exposed to the same food supply should respond similarly in regard to growth regardless of their growth history. Abalone translocated from slow growth to fast growth sites obtained the same k as the fast growth control population, reflected in the parallel lines of regression at both sites, but grew at a consistently slower rate for all sizes and so that there was a significantly smaller L_{∞} . This suggests that the growth history of an individual does not affect its future k but will affect its maximum size. This difference in average L_{∞} might be expected if there is an age effect on growth, given that slow growing individuals at any given size are likely to be older than fast growing abalone of the same size. An alternative hypothesis is that the previous history of abalone determines their resource allocation, so that the larger transplanted abalone may devote almost all of the resources they have available beyond maintenance requirements, to reproduction. It would be useful to study changes in growth and fecundity over longer periods to see if this pattern persists.

Direct comparison of translocated abalone with the thinning experiment controls could not be made because the latter were tagged in situ. Translocated abalone had to be handled on the boat so all controls were treated in the same way. When the growth of translocated abalone was compared with slow growth controls, the

TABLE 3.

Estimated asymptotic lengths and confidence intervals based on X axis intercepts of regressions for control and treatments at each site of the density reduction experiment.

Site	Treatment	n	-95% CI	L_{∞}	+95% CI
Aggregation	Control	213	149.2	153.9	158.7
	Thinned	142	135.5	142.1	148.6
Lighthouse	Control	99	137.8	144.1	150.5
	Thinned	74	137.6	143.3	149.1
Sand Gutters	Control	96	137.9	141.3	144.8
	Thinned	85	139.3	145.9	152.5

TABLE 4.

ANCOVA outputs for tagged abalone translocated from the Lighthouse and Aggregation sites compared to slow growth and fast growth controls.

Translocated from	Comparison	Source	DF	MS	F-ratio	P-value
Aggregation	Slow V Trans.	Interaction	1	126.743	19.650	<0.001
		Error	292	6.450		
Lighthouse	Slow V Trans.	Interaction	1	12.044	2.177	0.143
		Error	124	5.531		
Lighthouse	Slow V Trans.	Treatment	1	107.655	19.281	<0.001
		Error	125	5.584		
Aggregation	Fast V Trans.	Treatment	1	1609.648	199.701	<0.001
		Error	299	8.060		
Lighthouse	Fast V Trans.	Treatment	1	699.589	97.456	<0.001
		Error	222	7.179		

pattern of growth response was the same as for thinning and was similar among sites. On the basis of these results it is reasonable to speculate that the manipulation of density reduction and change of habitat have increased the quantity of food available to the individuals within that population, and this led to the size-specific growth response seen in both experiments over the short periods of this study.

The magnitude of the response in shell growth to the density reductions of 40% to 70% in unfished populations is critical for stock assessment. Many biomass dynamic fishery models have adopted sustainability criteria such as failure to maintain a mini-

mum proportion of original biomass, to trigger management intervention. For these triggers the values used are usually around 40%, equivalent to a loss of 60% of the original unfished biomass (Smith & Smith 2002). For abalone, Shepherd & Baker (1998) suggested that a minimum egg production of 40% to 50% be maintained. Depending on size limits, these values are likely to equate to density reductions within the range of this study. Thus it is important that assessments of areas are not carried out soon after a small area is fished. The magnitude of the density dependent growth response we observed, despite the short-term nature of our experiment, suggests that biomass reduction of legal sized abalone by fishing would be at least partly compensated by growth over longer periods.

In the longer term, density dependent growth would accelerate stock declines in fisheries with a legal minimum length (LML), because fishing would increase the growth rate of sublegal sized abalone, rendering them vulnerable to fishing after a shorter period of time. Density dependent growth becomes compensatory however, when the increase in size reduces mortality or increases fecundity (Rose et al. 2001). For *H. laevigata* there is clear evidence of both size dependent mortality (Shepherd & Breen 1992) and rapidly increasing fecundity with increasing size (Shepherd et al. 1992). Therefore the significant increases in growth rate that we observed could be expected to act as a regulatory mechanism that promotes increased population growth to compensate for losses caused by density reduction.

McAvaney et al. (this volume) have shown that juveniles at low density will reach larger sizes and mature earlier than juveniles at high density. Their study involves the manipulation of densities of

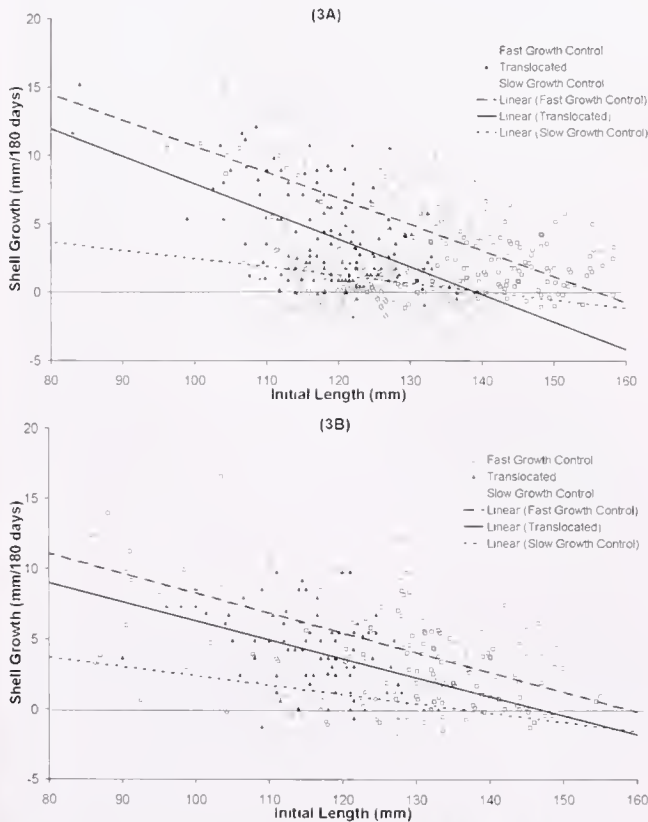


Figure 3. Regression of growth increment versus initial size for relevant fast and slow growth control populations compared with abalone translocated from the (A) Lighthouse site and (B) Aggregation site.

TABLE 5.

Estimated asymptotic lengths and confidence intervals based on regressions of control and treatment data at each site of the translocation experiment.

Site	Treatment	n	-95% CL	L_{∞}	+95% CL
Aggregation	Fast control	174	151.1	156.5	162.0
	Translocated	135	133.6	139.7	145.9
	Slow control	165	133.4	138.1	142.7
	Fast control	141	153.4	158.9	164.4
Lighthouse	Translocated	84	141.1	146.8	152.5
	Slow control	461	133.4	137.6	141.7

juvenile greenlip abalone (<90 mm) outplanted into the wild. It is important to determine whether a reduced density of adult sizes leads to faster growth of juveniles that occupy more cryptic habitats and thus might be more affected by juvenile than by adult density.

In our study we have shown that compensatory growth will occur in fished abalone populations. We hypothesize that larger abalone were allocating extra resources into reproduction and that if this were the case, further compensatory effects would occur. If fishing large adults leads not only to faster growth of small adults but also to faster recruitment of adults from the juvenile size classes, then these mechanisms in combination may assist to stabilize adult stocks against the impact of fishing and at least partly maintain the egg production capacity of abalone populations, which will in turn increase prospects for their sustainability.

ACKNOWLEDGMENTS

The authors thank the Central Zone divers and deckhands of South Australia for their contribution in the field; Michael Tokley and Bob Pennington for administrative assistance; Sylvain Huchette for the very valuable assistance in experimental design and lead role in fieldwork. The authors also thank Harry Gorfine and Matt Reardon for constructive criticism of the manuscript; Scoresby Shepherd, James Brook, Simon Hart, Matt Reardon, Thor Saunders, Steven Mayfield, and Brian Davies for their assistance in field work. This research was conducted as a component of a project established between the University of Melbourne and the Abalone Industry Association of South Australia, funded under the Australian Research Council (ARC) Strategic Partnerships with Industry Research and Training (SPIRT) Scheme

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Photographed at Dalian Zinda Sea Food Co, October 2003, during the post-conference tour after the 5th International Abalone Symposium, China. (Photo by Rob Tarr, Marine and Coastal Management, Cape Town, South Africa.)

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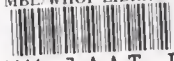
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